# Extracellular acid proteases of wine microorganisms: gene identification, activity characterization and impact on wine

by

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# Declaration

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# Summary

Non-*Saccharomyces* yeasts of oenological origin have previously been associated with spoilage or regarded as undesired yeasts in wine. However, these yeasts have recently come under investigation for their positive contribution towards wine aroma especially when used in sequential or co-inoculated fermentations with *Saccharomyces cerevisiae*. These yeasts are also known to secrete a number of enzymes that could be applicable in wine biotechnology. Amongst these enzymes are aspartic proteases. The secreted proteases from some non-*Saccharomyces* yeast may play a role in protein haze reduction, as demonstrated by some authors, while simultaneously increasing the assimilable nitrogen content of the wine for the utilization and growth of fermentative microorganisms. Moreover, the proteases may have an indirect effect on wine aroma by liberating amino acids that serve as aroma precursors. Although many screenings have been performed detecting protease activity in non-*Saccharomyces* yeasts, no attempts have been made to characterize these enzymes. This study set out to isolate and characterize genes encoding extracellular aspartic proteases from non-*Saccharomyces* yeasts.

An enzymatic activity screening of a collection of 308 *Saccharomyces* and non-*Saccharomyces* yeasts, isolated from grape must, was performed. The aspartic protease-encoding genes of two non-*Saccharomyces* yeasts, which showed strong extracellular proteolytic activity on plate assays, were isolated and characterized by *in silico* analysis. The genes were isolated by employing degenerate and inverse PCR. One gene was isolated from *Metschnikowia pulcherrima* IWBT Y1123 and named *MpAPr1*. The other putative gene was isolated from *Candida apicola* IWBT Y1384 and named *CaAPr1*. The *MpAPr1* gene is 1137 bp long, encoding a 378 amino acid putative protein with a predicted molecular weight of 40.1 kDa. The *CaAPr1* putative gene is 1101 bp long and encodes a 367 amino acid putative protein with a predicted molecular weight of 39 kDa. These features are typical of extracellular aspartic proteases. The deduced protein sequences showed less than 40% homology to other yeast extracellular aspartic proteases. By heterologous expression of *MpAPr1* in *S. cerevisiae*, it was confirmed that the gene encodes an extracellular acid protease. The expression of *MpAPr1* was shown to be induced in media containing proteins as sole nitrogen source and repressed when a preferred nitrogen source was available. The gene was expressed in the presence of casein, bovine serum albumin (BSA) and grape juice proteins and repressed in the presence of ammonium sulphate. Expression was most induced in the presence of grape juice proteins, which was expected since these proteins are present in the natural habitat of the yeast. A genetic screening confirmed the presence of the *MpAPr1* gene in 12 other *M. pulcherrima* strains isolated from grape juice. The extracellular protease activity of the strains was also visualized on plates. As far as we know, this is the first report on the genetic characterization of secreted aspartic proteases from non-*Saccharomyces* yeasts isolated from grape must and provides the groundwork for further investigations.

# Opsomming

Nie-*Saccharomyces* giste is voorheen met wynbederf geassosieer en hul teenwoordigheid in wyn is ongewens. Hierdie giste is onlangs ondersoek vir hulle positiewe bydrae tot wyn aroma, in veral sekwensiële en ko-inokulerings met *Saccharomyces cerevisiae*. Sommige van die nie-*Saccahromyces* giste skei ‘n verskeidenhied ensieme af wat moontlik vir die wynmaker van nut kan wees. Een groep van hierdie ensieme is die aspartiese suurproteases. Soos deur sommige navorsers aangetoon word, kan die proteases die vorming van proteïenwaasverlaging, terwyl dit terselfdertyd die assimilerende stikstofinhoud van die wyn vir die gebruik en groei van fermentasie-mikroörganismes verhoog. Die proteases kan moontlik ook ‘n indirekte uitwerking op die aromaprofiel van die wyn hê deur die vrystelling van aminosure wat as aromavoorlopers dien. Alhoewel baie studies gedoen is wat die ekstrasellulêre teenwoordigheid van proteases bevestig in nie-*Saccharomyces* giste wat van druiwesap/wyn afkoms is, is daar geen dokumentasie oor die genetiese karakterisering van hierdie ensieme beskikbaar nie. Die doel van hierdie studie was om gene wat aspartiese proteases in nie-*Saccharomyces* giste enkodeer, te isoleer en gedeeltelik te karakteriseer.

‘n Versameling van 308 *Saccharomyces* en nie-*Saccharomyces* giste wat uit druiwe sap geïsoleer is, is gesif vir ensiematiese aktiwiteit deur plaattoetse uit te voer. Twee gene wat aspartiese protease enkodeer, is geïsoleer van twee nie-*Saccharomyces* giste. Dit hetpositief gedurende die aktiwiteitstoetse getoets en is deur *in silico*–analise gekarakteriseer. Die gene is deur die uitvoering van gedegenereerde en inverse PKR geïdentifiseer. Een geen is vanaf *Metschnikowia pulcherrima* IWBT Y1123 geïsoleer en is *MpAPr*1 genoem, terwyl die ander van *Candida apicola* IWBT Y1384 geïsoleer en *CaAPr1* genoem is. Die *MpAPr*1-geen is 1137 bp lank en enkodeer ‘n proteïen wat uit 378 aminosure bestaan met ‘n voorspelde molekulêre massa van 40.1 kDa. Daar teenoor is die *CaAPr1*-geen 1101 bp lank en enkodeer vir ‘n proteïen wat uit 367 aminosure met ‘n molekulêre massa van 39 kDa bestaan. Hierdie eienskappe is kenmerkend van aspartiese protease. Die afgeleide proteïenvolgorde het minder as 40% homologie met ander ekstrasellulêre aspartiese proteases vertoon, wat dui op die nuwigheid van hierdie ensieme. Die *MpAPr1*-geen is heterologies in *S. cerevisiae* YHUM272 uitgedruk en dit het bevestig dat die geen inderdaad ‘n ekstrasellulêre aspartiese protease enkodeer. Die *MpAPr1*-geen is uitgedruk in media wat alleenlik proteïen as stikstofbron bevat het, terwyl dit onderdruk is in gevalle waar ‘n verkose stikstofbron beskikbaar was. Die geen is uitgedruk in die teenwoordigheid van kaseïen, BSA en proteïene afkomstig vanaf druiwesap en in die teenwoordigheid van ammoniumsulfaat onderdruk. Die hoogste uitdrukking was in die teenwoordigheid van druifproteïene. Hierdie proteïene is teenwoordig in die natuurlike habitat van die gis en is dus dalk ‘n bekende stikstofbron vir die gis. ‘n Genetiese sifting het die teenwoordigheid van die *MpAPr1*-geen in 12 ander *M. pulcherrima*–rasse, wat ook van wynkundige oorsprong is, bevestig. Die aspartiese protease-aktiwiteit van die 12 rasse is ook op agarplate waargeneem. Na ons wete, is dit die eerste verslag oor die genetiese karakterisering van afgeskeide aspartiese proteases van nie-*Saccharomyces* giste van wynkundige oorsprong en verskaf die grondslag vir verdere ondersoek.

This thesis is dedicated to

**My Mother**

# Biographical sketch

Vernita Reid was born in Bloemfontein, South Africa on the 12th of November 1984. She attended Heide Primary School and completed her matriculation at Oranje Girls' School in 2002. She obtained a BSc degree in Food Biotechnology in 2007 and a BSc Honours degree in Food Science in 2008 from the University of the Free State. She enrolled at Stellenbosch University in 2010 for an MSc in Wine Biotechnology.

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# Preface

This thesis is presented as a compilation of four chapters.

**Chapter 1** **General Introduction and project aims**

**Chapter 2** **Literature review**

Aspartic proteases and non-*Saccharomyces* yeasts and their potential application in wine biotechnology

**Chapter 3** **Research Results**

Identification and characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384

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# Chapter 1

## General introduction and project aims

# General introduction and project aims

## 1.1 Introduction

The production of wine is a complex biochemical transformation facilitated by a large pool of enzymes of plant and microbial origin (Pretorius et al., 1999; Fleet, 2003). The yeast *Saccharomyces cerevisiae* plays the predominant role in the transformation of grape juice to wine, whether the juice is inoculated with commercially available *S. cerevisiae* strains or left to ferment spontaneously with the microorganisms present in the grape must (Fleet et al., 1984; Bisson, 2004). This yeast has high ethanol tolerance and fermentation capacity and releases secondary metabolites which plays a role in enhancing the aroma and flavour of wine. The metabolic activities of this yeast are very well characterized (Fleet, 2003). Besides *S. cerevisiae,* a range of other yeast species are also present in spontaneously fermenting must and some may also be present in wine. These yeasts, classified as non-*Saccharomyces* yeasts, were thought to be detrimental to wine flavour and aroma and were mostly categorized as wine spoilage yeasts (Du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003). These include yeasts of the genera *Candida*, *Metschnikowia*, *Debaryomyces, Zygosaccharomyces, Kluyveromyces,* and *Kloeckera,* to name a few (Fleet et al., 1984; Heard and Fleet, 1987). However, it has been demonstrated that some of these yeasts can confer desirable aroma nuances to wine when used in conjunction with *S. cerevisiae* in co-inoculated fermentations (Ciani and Comitini, 2011; Domizio et al., 2011). It has also been reported by a number of authors that some non-*Saccharomyces* yeasts are good secretors of extracellular enzymes e.g. pectinases, glucosidases and proteases, that could be of interest to the winemaker (Charoenchai et al., 1997; Fernandez et al., 2000; Strauss et al., 2001). Of particular interest are the extracellular proteases produced by some non-*Saccharomyces* wine yeasts.

It has already been reported in literature that the addition of proteases to wine is efficient for reducing protein haze formation without being detrimental to wine quality (Lagace and Bisson, 1990; Pocock et al., 2003). Protein haze formation in white wine is usually due to the denaturation of wine proteins during bottle storage (Hsu et al., 1987; Ferreira et al., 2001; Pocock and Waters, 2006; Marangon et al., 2011). The presence of haze reduces the commercial value of the wine, making it unacceptable for consumers as it may be perceived as microbial spoilage (Pocock and Waters, 2006). Winemakers usually add bentonite to their white wine in order to precipitate the proteins down before bottling. The disadvantages are that such a treatment is expensive, reduces product yield and may have a negative effect on wine aroma (Waters et al., 2005). Besides the potential to reduce unsightly protein haze in white wine, proteases may also liberate peptides and amino acids thereby increasing the assimilable nitrogen content of wine for the growth of fermentation (and spoilage) microorganisms, which is essential for efficient fermentation. An increase in assimilable nitrogen may also lead to an

increase in the formation of aroma compounds such as ethyl acetate, acetic acid and other volatile acids (Bell and Henschke, 2005).

### 1.2 Rationale and scope of the study

Wine is a unique environment that is characterized by a low pH (2.8 – 4.2) (Somers, 1971), low temperature (15 - 25˚C), and the presence of inhibitors such as $SO_2$ (160 mg/l), ethanol (10 – 25%) and low sugar content (2.5 – 12 g/l). Organisms and their secretome that are able to survive or even flourish under these conditions are highly adapted. Certain non-*Saccharomyces* yeasts that are able to survive in wine also have the ability to secrete enzymes into the wine matrix (Bossi et al., 2006). Investigations have been conducted demonstrating the production of extracellular acid proteases by wine non-*Saccharomyces* yeasts (Charoenchai et al., 1997; Fernández et al., 2000; Strauss et al., 2001) but none have focused on characterizing these enzymes on genetic level or the mechanism involved in the secretion (and regulation) of these enzymes. The wealth of knowledge and potential regarding non-*Saccharomyces* yeasts with hidden potential for oenology is largely untapped.

The aim of this study is to identify and characterize extracellular acid protease encoding genes from non-*Saccharomyces* yeast isolated from grape must. Part of the focus of this work is to better understand the adaptation and the interactions of these microorganisms in the particular life medium that wine is. It would contribute to the global knowledge of the potential certain wine microorganisms might possess to survive in wine. The study will provide further insight into these enzymes on genetic and activity levels.

**Specific objectives of the study**

1. To identify and isolate new genes encoding aspartic proteases from non-*Saccharomyces* yeasts isolated from grape must
2. To characterize the genes and the proteins that they encode
3. To explore the potential applicability of these enzymes in winemaking

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# Chapter 2

# Literature review

## Aspartic proteases of non-*Saccharomyces* yeasts and their potential application in wine biotechnology

# 2. Literature review

## 2.1 General introduction

Winemaking involves the biochemical conversion of grape juice to wine where microorganisms, mainly yeasts, in the juice convert glucose to ethanol, carbon dioxide and a range of other secondary metabolites (Fleet, 2003). The conversion is facilitated by a large pool of enzymes of both plant and microbial origin. Winemakers reinforce this pool of indigenous enzymes by adding a variety of industrially produced enzymes such as pectinases, hemicellulases, glucanases and glycosidases in order to help enhance clarification, juice yield, as well as the release of aroma compounds, tannins and colour.

The yeast *Saccharomyces cerevisiae* is the predominant yeast responsible for fermentation (Fleet et al., 1984). In recent years however, researchers have been investigating the impact non-*Saccharomyces* yeasts have on wine production. Some of the non-*Saccharomyces* yeasts have been shown to secrete hydrolytic enzymes including proteases that might be of interest to the winemaker (Esteve-Zarzoso et al., 1998; Dizy and Bisson, 2000; Fernández et al., 2000).

This review consists of three main sections. The first will focus on aspartic proteases with a short introduction into proteolytic enzymes followed by a more detailed discussion into aspartic proteases, i.e. their structure, catalytic mechanisms and the secretion of extracellular aspartic proteases in yeasts. The last two sections will discuss the oenological importance and the role of non-*Saccharomyces* yeasts in winemaking, and the potential of aspartic proteases in winemaking, respectively.

## 2.2 Proteolytic enzymes

Proteolytic enzymes catalyse the cleavage of peptide bonds within peptides and proteins. They are encoded by about 2% of genes in all kinds of organisms. These enzymes regulate most physiological processes (Tyndall et al., 2005). Some of the important medical roles that proteolytic enzymes fulfil include food digestion, protein turnover, blood coagulation, embryonic development and cell division. Approximately 14% of the five hundred human peptidases are under investigation as drug targets and include the β-secretase that play a role in Alzheimer's disease. The human immunodeficiency virus (HIV) protease is another well-known drug target. They are thus an important group of enzymes in scientific, medical research and biotechnology (Rawlings et al., 2009).

### 2.2.1 Definition and categorization of proteolytic enzymes

Proteolytic enzymes are also known as proteinases or proteases, however the Enzyme Commission (EC) and the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) prefer the term peptidases be used for all enzymes that

hydrolyse peptide bonds (subclass E.C.3.4). Nevertheless, proteolytic enzymes are perhaps the most generally understood term in the current usage. Exopeptidases cleave one or a few amino acids from the N- or C-terminus while endopeptidases act internally in the polypeptide chains. Exopeptidases that hydrolyse at a free N-terminus to release a single amino acid residue are called aminopeptidases, while those that release dipeptides and tripeptides are named dipeptidyl-peptidases and tripeptidyl-peptidases, respectively. Those hydrolysing at a free C-terminus to release a single residue are named carboxypeptidases and those releasing dipeptides are named peptidyl-dipeptidases. Other exopeptidases remove terminal residues that are substituted, cyclized or linked by isopeptide bonds (peptide linkages other than those of α-carboxyl to α-amino groups) (Barrett et al., 1998).

Proteases are categorized based on their catalytic mechanism, the amino acid residues present in the catalytic site and their three-dimensional structure. According to the NC-IUBMB, proteases can be categorized into four mechanistic classes which include the serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases and metallo-endopeptidases. Each type of protease is specific in its ability to break a certain peptide bond and exhibits a characteristic set of functional amino acid residues arranged in a specific configuration to produce its catalytic site (Barrett et al., 2004; Tyndall et al., 2005). Table 2.1 shows the different protease families, some common examples and the amino acid residues present in each catalytic domain. Proteases commonly recognize the extended or *α*-strand backbone conformation in substrates, inhibitors, and products (Tyndall et al., 2005).

The MEROPS database is a manually curated information resource dedicated solely to peptidases, their substrates and inhibitors. It can be found at http://merops.sanger.ac.uk. The MEROPS database divides peptidases into protein species which are then sub-divided into families according to statistically significant similarities in their amino acid sequences. Homologous families are then grouped into clans.

The protein species are the Aspartic peptidases, Cysteine peptidases, Glutamic peptidases, Metallopeptidases, Asparagine peptidases, Serine peptidases, and Threonine peptidases.

The Serine proteases have the catalytic triad aspartic acid, histidine, and serine and play important roles in digestion. They have one of two structural folds: the trypsin-like type (serine protease I) which is made up of two β-barrels and the subtilisin-like type (serine protease II) made up of a three-layer αβα sandwich fold.

The Cysteine proteases have similar folds as the serine type but are more V-shaped and have the catalytic dyad histidine and cysteine or triad with an aspartic acid residue. A common example is papain which is used as a meat tenderiser.

The Metalloproteases have a characteristic divalent zinc metal ion in their catalytic site and are important for wound healing and tissue morphogenesis (Rao et al., 1998).

The Aspartic proteases, which will be the main focus of this review and in particular those secreted by non-*Saccharomyces* yeasts, have a tertiary structure consisting of two approximately symmetric lobes with each lobe carrying an aspartic acid residue to form the catalytic site. Unlike the other types of proteases, the activity of the aspartic proteases is dependent on low pH conditions (Northrop, 2001; Cascella et al., 2005; Borelli et al., 2008).

Threonine proteases contain a threonine nucleophile at their N-terminus and sometimes a serine residue as well. Glutamic proteases, which were formerly known as pepstatin-insensitive carboxyl proteases, have a glutamic acid and a glutamine residue in their catalytic sites. They are also active at acidic pH and are found in some bacterial and fungal species (Tanokura et al., 1992; Fujinaga et al., 2004; Tyndall et al., 2005).

Asparagine proteases were recently discovered and are found in certain pathogenic viruses and bacteria (e.g. *Escherichia coli*) (Rawlings et al., 2011). The catalytic site may consist of a single residue, asparagine or may contain asparagine with serine, asparagines or cysteine.

Besides these families there have been discoveries of proteases with unidentified catalytic mechanism. This indicates that novel types of proteases may exist (Tanokura et al., 1992; Tyndall et al., 2005; Rawlings et al., 2009).

### 2.2.2 Aspartic proteases

#### 2.2.2.1 General description

Aspartic endopeptidases (E3.4.23.x) are widely distributed in living organisms from vertebrates to fungi, plants and retroviruses. Most of these enzymes are composed of approximately 323 to 340 amino acid residues, with molecular weights ranging between 35 000 to 50 000 Daltons (Da) and isoelectric points (pI) ranging between 3 and 4.5 because of the high percentage of acidic amino acid residues (about 13%) in the proteins. They have optimum function at pH 3 to 4. They show substrate specificity towards extended peptide substrates and residues with large hydrophobic side chains on either side of the scissile bond (Barrett et al., 1998; Rawlings et al., 2009).

**Table 2.1** Families of proteolytic enzymes.

| Family | Representative protease(s) | Characteristic active site residues | Optimal pH | Inhibitors |
|---|---|---|---|---|
| Serine protease I | Chymotrypsin<br>Trypsin<br>Elastase | $Asp^{102}$, $Ser^{195}$, $His^{57}$ | Neutral and alkaline (7-11) | PMSF |
| Serine protease II | Subtilisin | $Asp^{32}$, $Ser^{221}$, $His^{64}$ | Neutral and alkaline (7-11) | PMSF |
| Cysteine proteases | Papain<br>Actinidin<br>Cathepsins B and H | $Cys^{25}$, $His^{159}$, $Asp^{158}$ | Neutral | Sulfhydryl agents (PCMB) |
| Aspartic proteases | Penicillopepsin<br>*Rhizopus chineses* acid proteases<br>Rennin | $Asp^{11}$, $Asp^{213}$ | Acid to neutral (2.5 – 7) | Pepstatin, EPNP, DAN |
| Metallo – proteases I | Bovine carboxypeptidase A | $Zn,Glu^{270}$, $Try^{248}$ | Neutral to alkaline (7 -9) | EDTA |
| Metallo – proteases II | Thermolysin | $Zn, Glu^{143}$, $His^{231}$ | Neutral to alkaline (7 -9) | EDTA |
| Threonine proteases | Polycystin-1 | $Thr^{3049}$ | Neautral | DON, |
| Glutamic proteases | Scytalidoglutamic peptidase | $Gln^{107}$, $Glu^{190}$ | Acid (2 – 6) | EPNP |
| Asparagine | Nodavirus peptidase | $Asp^{75}$,$Asn^{363}$ | Neutral | Unknown |

DAN, diazoacetylnorleucinemethyl; DON, 5-diazo-4-oxonorvaline; PMSF, phenylmethylsulfonyl fluoride; PCMB, (*p*-chloromercuribenzoic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane); EDTA Ethylenediaminetetraacetic acid. (Beynon and Bond, 1990; Rao et al., 1998).

As stated previously, these enzymes are characterized by the presence of two aspartic acid side chains in the catalytic site. They are inhibited by pepstatin, a hexapeptide from *Streptomyces* which contains the unusual amino acid statine (Davies, 1990; Dunn, 2002). Examples of aspartic proteases (APs) include rennet which has been used for thousands of years in cheese making, cathepsin D, a major lysozomal enzyme and rennin which plays an important role in blood pressure. Pepsin, a gastric enzyme, is probably the most studied AP and was the second protein structure to be analyzed by X-ray diffraction (Bernal and Crowfoot, 1934). Pepsin has often been used as a model for the study of APs. The APs of retroviruses such as Rouse Sarcoma and HIV have also been studied extensively and their crystal structures have been determined as early as 1989 (Navia et al., 1989). APs play an important role in sporulation of fungi (Davies, 1990). According to the MEROPS and Protein Data Bank (PDB), there are eight sub-families within the Aspartic proteases with the sequence Asp-Thr(Ser)-Gly at their active site. The subfamilies differ according to the specific residues in the active site, the position of the catalytic aspartic acid residues in the peptide chains, substrate

specificity, the number of disulfide bridges in their structure (Rawlings and Bateman, 2009; Rawlings et al., 2009) and the optimal pH at which the enzymes function.

#### 2.2.2.2 Structure of aspartic proteases

The pepstatin-sensitive aspartic protease family can be divided into two fold families: the eukaryote pepsin-like type and the retroviral type (Figure 2.1) (Dunn, 2002). In pepsin-like aspartic proteases the tertiary structure consists of two asymmetric lobes formed by α/β monomers. A catalytic aspartic dyad is formed at the lobe interface with an aspartic residue contributed by each lobe or domain. A flap made up of a β-hairpin covers the catalytic site. The interface or bridge between the two lobes is a six-stranded, antiparallel β-sheet. The active site cleft within the lobes is large enough to accommodate approximately seven amino acid residues. The retroviral types on the other hand are β homodimers; aspartates are located on two loops at the monomer interface and two β-hairpins cover the active site (Sielecki et al., 1991; Dunn, 2002). The amino acid residues in eukaryotes are generally Asp-Thr-Gly-Ser/Asp-Ser-Gly-Thr and Asp-Thr-Gly-Ala in retroviral proteases. The two families are evolutionary related and it appears that the eukaryotic APs evolved from the prokaryotic APs as a result of gene duplication: the cleavage site lobes are homologous, the aspartic dyad is situated at the interface region of the lobes for both families, and the viral subunits are structurally similar to the N-terminal lobes of the pepsin-like enzymes. A water molecule forms part of the catalytic site and is located between the aspartic residues, binding them (Blundell et al., 1990; Andreeva et al 2006; Cascella et al., 2005).

As far as sequence and domain similarities are concerned, Cascella and co-workers (2005) investigated the structures and amino acid sequences of aspartic proteases and found that approximately 20% of the protein sequences of the pepsin family are conserved or show highly conserved mutations, and approximately 80% of these residues fall within three regions. The first region is the catalytic site region made up of the two aspartate-containing loops and β-sheets located at the lobes' interface. The second region comprises four anti-parallel β-sheets located opposite the substrate binding site. This is the only structural region that cross-links the two lobes of the protein. The third conserved region is located on the surface of the N-terminal lobe. It is mostly comprised of polar residues (particularly serine, threonine and aspartate) involved in specific contacts on the protein surface.

The enzyme fold is very well maintained; the core is rigid whereas the solvent-exposed loops are mobile. A fully conserved tyrosine residue is located opposite the active site which is believed to be responsible for clamping the substrate. The number and position of disulfide bridges may vary from sequence to sequence and may impact more strongly on native-state stability (Cascella et al., 2005; Friedman and Caflisch, 2010).

There are seventeen conserved water molecules within the structure of aspartic proteases of higher organisms (Chitpinityol et al., 1997; Andreeva et al., 2006). They facilitate stabilization of the fold and enable the rigidity of the active site conformation.

#### 2.2.2.3 Catalytic mechanism of aspartic proteases

The vertebrate aspartic proteases, and it is believed most of the fungal aspartic proteases, are synthesized as inactive zymogens, and contain an additional N-terminal segment approximately 45 amino acid residues long, that gets cleaved and separated upon activation (Davies, 1990). The pro-enzyme of pepsin is comprised of one β-strand and three α-helices (Dunn, 2002) and contains a number of basic amino residues including nine lysine, two arginine and two histidine residues (James and Sielecki, 1986). It occupies the active site cleft at the interface where the two lobes meet. Autocatalytic activation, i.e. self-cleavage of the pre-pro-segment, occurs upon exposure to an acidic environment. As the pH is decreased, acidic residues get protonated and this disturbs the electrostatic interactions with positively charged amino acid residues on the propeptide. Activation can be done by intermolecular or intramolecular activation. Between pH 4 and 5, intermolecular cleavage dominate, while at lower pH, activation tends to be intramolecular (Campos and Sancho, 2003). The flap residues undergo major displacements upon ligand binding.

Two catalytic mechanisms have been proposed. The first catalytic mechanism is a general acid-general base mechanism where the aspartate carboxyl groups act alternately as general acid and general base. Following exposure to low pH, cleavage events occur that lead to a conformational rearrangement. Optimal pH is between 3 and 4. A water molecule is hydrogen bonded to the two aspartic residues and acts as the nucleophile that attacks the carbonyl carbon of a scissile peptide bond arranged in the active site. Simply, the reaction proceeds in two chemical steps of similar free energy barriers ($k_{cat}$ ~ 18 kcal/ mol). Using pepsin as example, in step 1 $Asp^{215}$ acts as a general base to remove one proton from the water molecule followed by nucleophilic attack of the water molecule to the carbonyl carbon of the substrate scissile bond while $Asp^{32}$ donates a proton to the carbonyl oxygen atoms of the scissile peptide bond. A tetrahedral intermediate is formed with $Asp^{215}$ being hydrogen bonded to the attacking oxygen atom, while the hydrogen remaining on that oxygen is hydrogen bonded to the inner oxygen of $Asp^{32}$. During step 2, a reversal of configuration occurs around the nitrogen atom of the scissile bond with the transfer of the hydrogen from $Asp^{215}$ to that nitrogen atom. At the same time a proton is transferred from the inner oxygen of $Asp^{32}$ to the carbonyl oxygen on the peptide bond being cleaved. Hereafter the C-N bond breaks releasing the two products. Asp215 is negatively charged at this stage and ready for the next round of catalysis (Dunn, 2002; Jiang et al., 2005; Coates et al., 2008). The mechanism is illustrated in Figure 2.2.

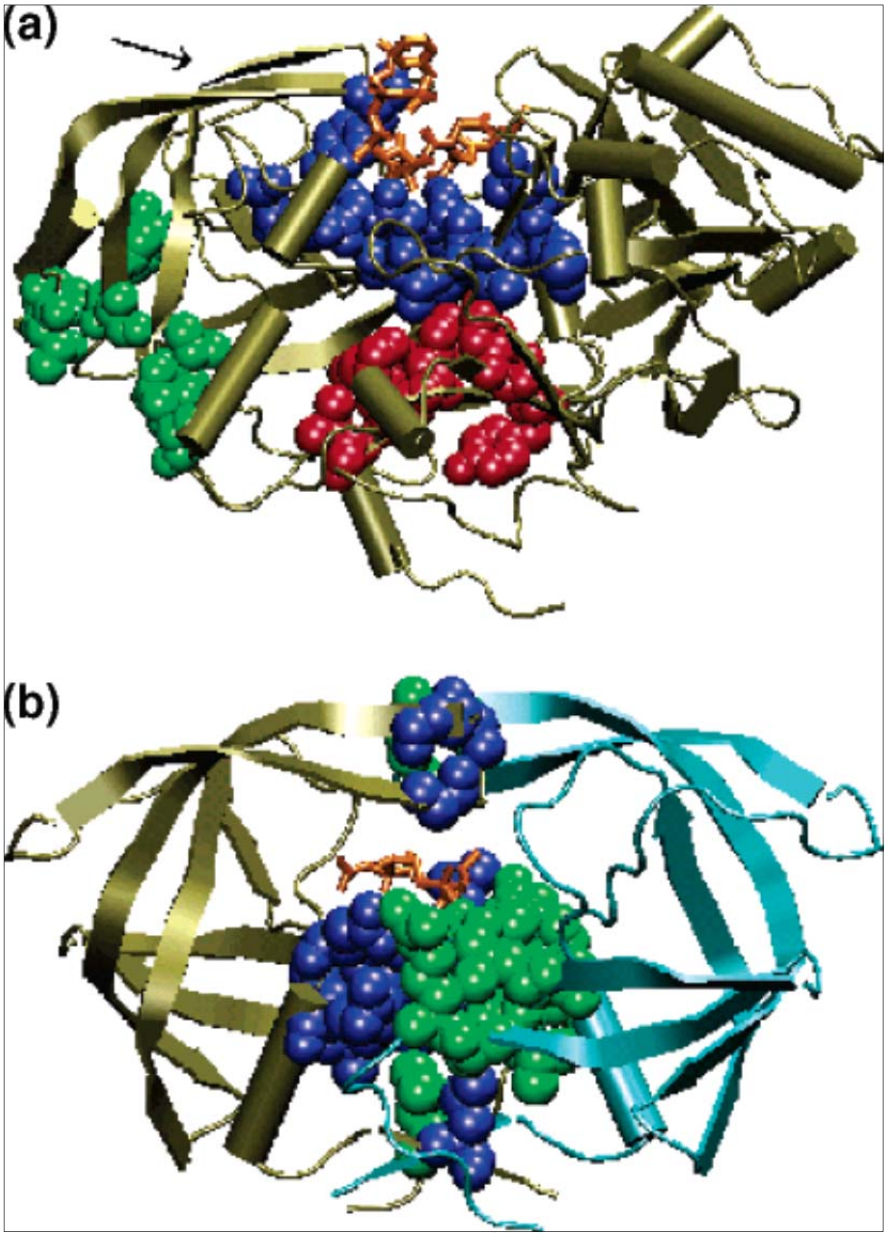


**Figure 2.1** The pepsin-like and retroviral protease folds. The structures of β-secretase (BACE) and human immunodeficiency virus (HIV-1) APs are shown in complex with model substrates (orange sticks). The conserved regions are drawn as spheres: (**a**) Eukaryotic β-secretase. Blue represents the β-hairpin, green represents the β-monomer and red represents the α-monomer. The black arrow indicates the flap position. (**b**) Retroviral HIV-1 AP. Green represents β-monomers and blue represents β-hairpins (Cascella et al., 2005).

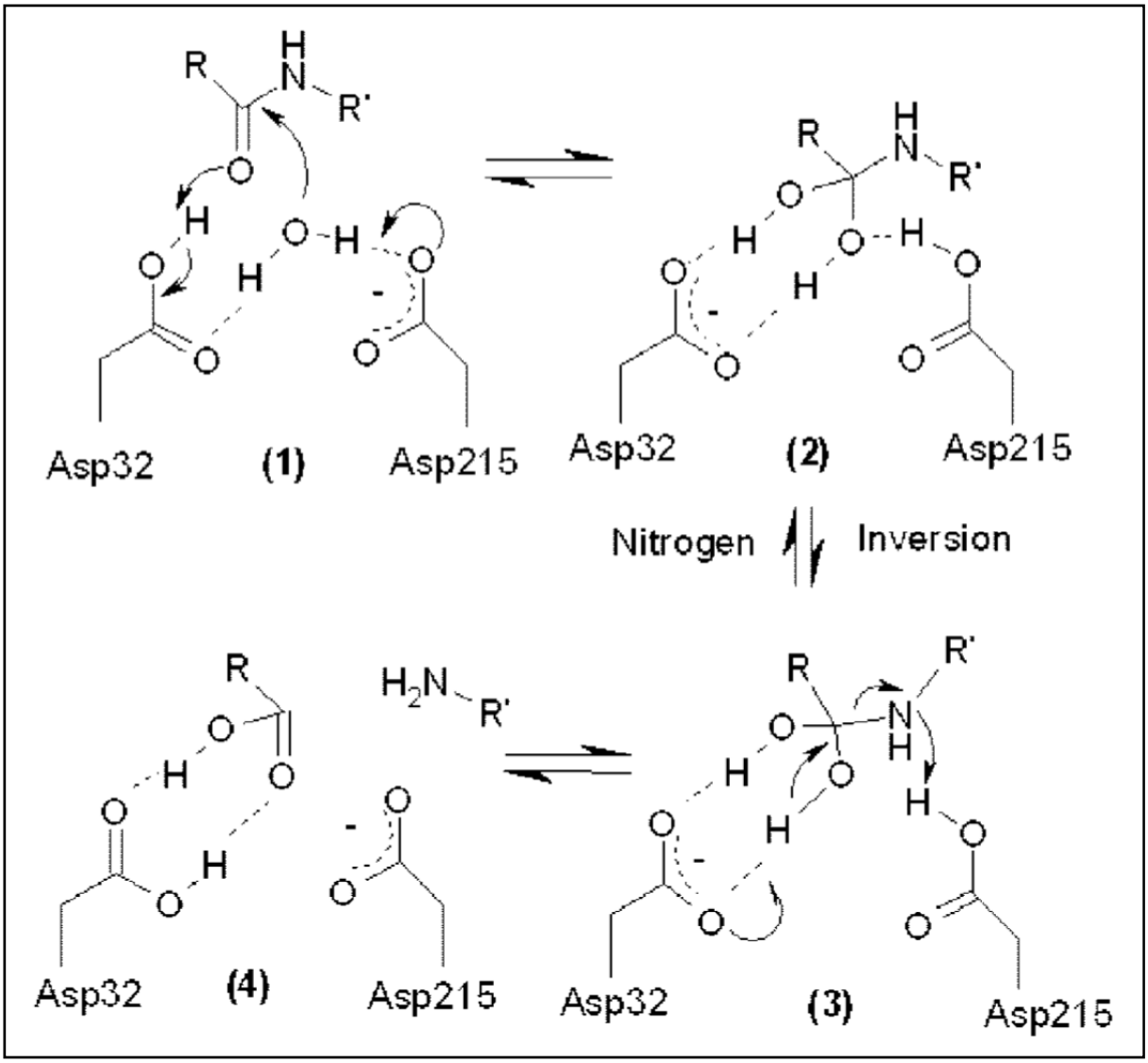


**Figure 2.2**. Catalytic mechanism of aspartic proteases. (1) The water molecule tightly bound to the aspartic residues nucleophilically attacks the scissile carbonyl bond. (2) The tetrahedral intermediate stabilized by hydrogen bonds to the negatively charged carboxyl of $Asp^{32}$. (3) and (4) Fission of the scissile C-N bond accompanied by transfer of a proton to the leaving amino group. Dashed lines indicate hydrogen bonds or charge sharing (as appropriate). (Coates et al., 2008).

The second mechanism has been proposed by Northrop (2001) who suggested that a low-barrier hygrogen bond (LBHB), not present in the first proposed mechanism, binds the two catalytic aspartic residues in the catalytic site (illustrated in Figure 2.3). In this mechanism, species E is the free enzyme poised for catalysis. Step 1 is the binding of substrate to form a loose complex (ES). Step 2 is the closing of the flap down upon the substrate to squeeze all components into the correct geometry and distances for the catalytic process to begin. Step 3 includes the removal of a proton from the bound water molecule to stimulate attack on the carbonyl carbon (FT). Step 4 involves a proton transfer to the nitrogen of the peptide bond (ET'). Step 5 is the bond cleavage event (EP'Q). Step 6 is the opening of the flap to free the products (FPQ) and step 7 is release of the products (FQ). Step 8 includes a loss of one proton (EQ') and step 9 involves binding of a new water molecule and re-formation of the low-barrier hydrogen

bond (E). This mechanism also differs from the previous one in that a final isomerisation step (step 9) is included which is not mentioned in the former mechanism. Some authors have disagreed with this proposal based on the wide angle between the two inner oxygens of the aspartic residues being too wide for a hydrogen bond to be formed (Andreeva and Rumsh, 2001; Dunn, 2002).

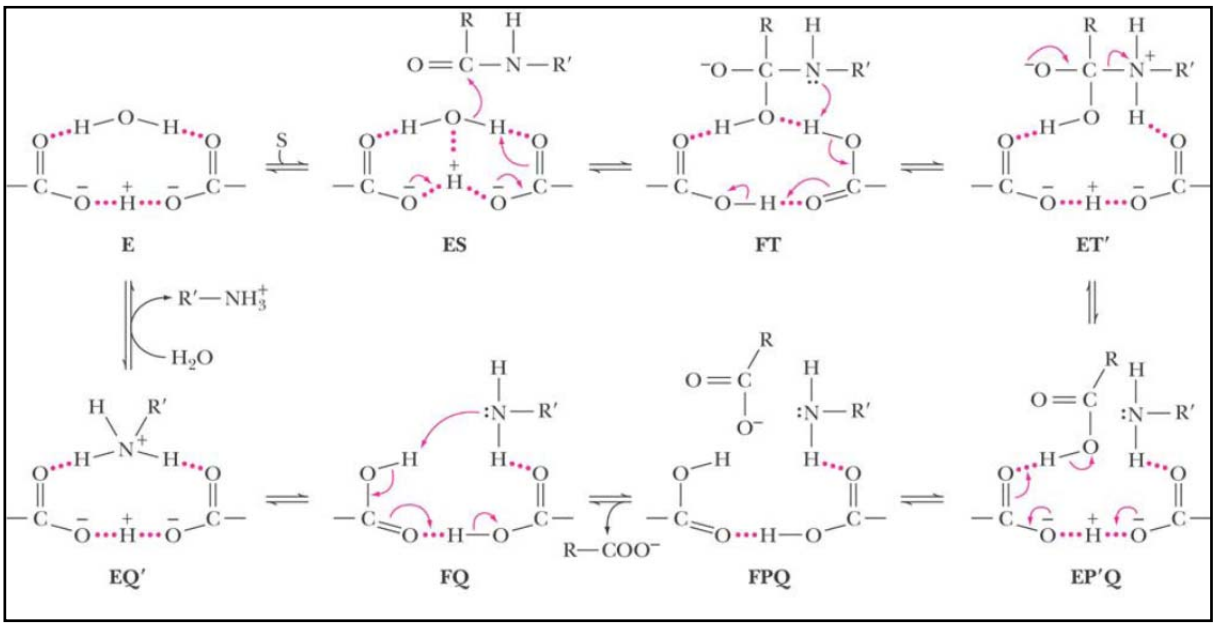


**Figure 2.3** The low-barrier hydrogen bond (LBHB) catalytic mechanism of aspartic proteases proposed by Northrop (2001).

### 2.2.2.4 Secretion pathway and expression in yeasts

The secretion pathway and processing of the secreted aspartyl proteases (also known as Saps) of the yeast genera *Candida* have been studied most extensively. Species *C. albicans*, *C. tropicalis* and *C. parapsilosis* are common human pathogens and cause oral and vaginal candidiasis. The Saps are virulence factors because they assist in penetration and invasion of the pathogen, provide nutrition to the cells and evade immune responses of the host (Naglik et al., 2003a). Ten *SAP* genes (*SAP1* – *SAP10*) have been identified in the *C. albicans* genome (Naglik et al., 2004).

The processing of the Saps is initiated with mRNA being transcribed in the nucleus and transferred to the cytoplasm followed by translation of a pre-pro-peptide on the rough endoplasmic reticulum (ER). The pre-pro-enzymes of *C. albicans* Saps is approximately 60 amino acids larger than the mature enzyme. A hydrophobic signal peptide on the N-terminus is recognized by signal recognition particles and receptors on the ER membrane and directs the protein into the secretion pathway (Naglik et al., 2003b; Cheng et al., 2008). The N-terminal signal peptide (pre-peptide sequence) is cleaved in the rough ER lumen by a signal peptidase

complex (von Heijne, 1985). After cleavage the signal peptide is rapidly degraded in the ER lumen. Once in the ER, the proteins are modified and folded through glycosylation and the formation of disulfide bonds. Glycosylation is the addition of glycans to the amino (N-glycosylation) or hydroxyl (O-glycosylation) groups of specific amino acid residues which aid in protein stability and function (Lehle et al., 2006). Thereafter, the pro-enzyme is transferred via vesicles to the Golgi apparatus where the pro-peptide region is cleaved by the Kex2 subtilisin-like endoproteinase which specifically cleaves peptides after a conserved lysine-arginine sequence (Togni et al., 1996; Newport and Agabian, 1997; Punt et al., 2003). Propeptides are about 20 amino acids long and carry one or two basic amino acid residues in their C-terminus and a few non-basic residues (Conesa et al., 2001; Naglik et al., 2003b). A simplified illustration of the pathway is shown in Figure 2.4. Alternative but less efficient processing pathways for Saps are thought to exist (Begga et al., 2000). Once activated, the enzyme is packaged into secretory vesicles and transported to the plasma membrane and either remains attached to the cell membrane, or is released into the extracellular space depending on the nature of the Sap.

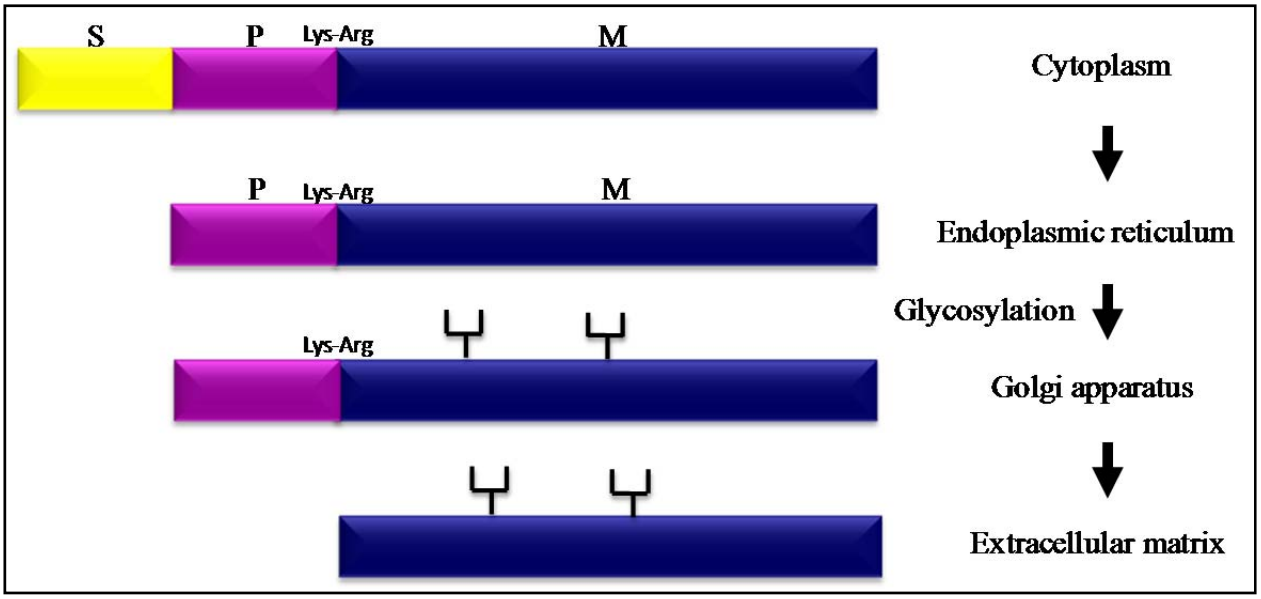


**Figure 2.4** Secretory pathway of secreted aspartyl proteases (Saps) of *Candida* spp.. S: Secretion signal peptide. P: Pro-peptide region. M: Mature protein. Ψ indicates glycosylation. Adapted from Togni et al. (1996).

Because the Sap enzymes are encoded as pre-pro-enzymes, regulation of proteinase expression is either controlled at mRNA transcription level or at protein level but occurs predominantly at the mRNA level (White and Agabian, 1995).

The maturation pathway of the extracellular alkaline protease, Aep of the yeast *Yarrowia lipolytica* has also been documented (Beckerich et al., 1998; Swennen and Beckerich, 2007). The pathway is relatively similar to that found in *Candida* spp. The acid protease of *Y. lipolytica,*

Axp however does not have a lysine-arginine signal site and is believed to follow a different maturation pathway yet to be elucidated (Beckerich et al., 1998; McEwen and Young, 1998).

### 2.2.3 Model systems of yeast proteases used in the food industry

*Y. lipolytica* is a yeast species known to secrete high amounts of a number of enzymes such as proteases, RNases and lipases (Peters and Nelson 1948; Tsugawa et al., 1969). It is a high producer of citric acid and has been used in enzyme secretion/expression studies as recombinant host because of its suitability for analysis by a range of molecular markers and molecular tools (Madzak et al., 2004; Yu et al., 2010). It is generally regarded as safe (GRAS) and has been used for biotechnological applications such as citric acid production, peach flavour production, and single cell protein production (Swennen and Beckerich, 2007). The acid proteases of *Y. lipolytica* have been investigated for use in beer brewing to reduce beer chill haze (Ogrydziak, 1993). Depending on the pH of the medium, the yeast either produces an alkaline or acid protease at the end of the exponential phase (Young et al., 1996; Swennen and Beckerich, 2007).

The protein and gene sequences of an acid protease secreted by the dimorphic hemiascomycetous yeast *Y. lipolytica* were determined by Young and co-workers (1996) and named Axp and *AXP1*, respectively. Axp is expressed upon carbon, nitrogen and sulphur starvation under acidic conditions (Young et al., 1996; Gonzalez-Lopez et al., 2001).

The Axp acid protease is secreted when the medium is at a pH range of 2.0 to 5.5 and has activity at pH 2-5. The enzyme has an optimum activity at pH 3.2. It is secreted as a precursor with a molecular weight of 42 kDa and undergoes autocatalytic activation at acidic pH. The mature form results from the cleavage of the bond located between phenylalanine-44 and alanine-45 between the C-terminus of the proregion and the N-terminus of the mature enzyme. The enzyme is non-glycosylated. As stated previously, Axp does not have a lysine-arginine signal site and it is believed that maturation occurs autocatalytically. Similar self-processing mechanisms have been described for *C. tropicalis* Sapt1, *Mucor miehei, M. pusillus and Aspergillus niger* var. *macrospores* aspartic proteases. It is known that Axp translocation occurs co-translationally (McEwen and Young, 1998).

The structural gene encodes a 397 residue long polypeptide including a 17 amino acid long signal peptide, a 27 amino acid long pro-region and the 353 amino acid long mature enzyme. According to the MEROPS database, the Axp enzyme belongs to the pepstatin-sensitive APs in family A1 and clan AA and has two potential disulfide bonds. Axp expression is regulated at transcriptional level by external pH (Beckerich et al., 1998; McEwen and Young, 1998; Gonzalez-Lopez et al., 2001; Rawlings et al., 2009).

Aspartic acid proteases are one of the most studied protease families. Because these enzymes are active under acidic conditions it is particularly useful for the food industry e.g. in cheese manufacturing and beer brewing. The aspartic acid proteases from a number of yeasts have been identified and are relatively well characterised e.g. the Saps of *C. albicans*. The same cannot be said for yeasts isolated from wine environments. Certain yeasts isolated from fermenting wine have been shown to have extracellular proteolytic activity which could be of use to the wine industry (Lagace and Bisson, 1990). The non-*Saccharomyces* yeasts found in grape juice have long been categorized as spoilage or unwanted yeasts in winemaking. However, in recent years these yeasts have come under investigation for their 'wine quality enhancing' potential, including the secretion of enzymes such as proteases (Charoenchai et al., 1997; Fernández et al., 2000; Jolly et al., 2006; Mendoza and Farías, 2010). These topics will be discussed in the following sections.

## 2.3 Oenological importance of non-*Saccharomyces* wine yeasts

### 2.3.1 Wine microbial diversity: Spontaneous and inoculated fermentations

During natural (spontaneous) fermentation of grape juice (or must) to wine, fermentable sugars are converted into alcohol, carbon dioxide and other by-products by microorganisms which include fungi, yeasts and bacteria, with the yeasts playing a predominant role (Pretorius et al., 1999; Fleet, 2003). The fermentation conversion occurs by a sequence of enzymatic reactions, both intra- and extracellularly of the different microbes. Enzymes originating from the grape itself are also involved. Grape must impose strong selective pressure on microorganisms due to its low pH, ~3.5, and high sugar content, typically 200-350 g/L, so that only certain microbial species are able to grow and survive in it (Ribereau-Gayon et al., 2006). The yeasts present during spontaneous fermentation may be divided into two groups, the *Saccharomyces* yeasts, particularly *S. cerevisiae* and the non-*Saccharomyces* yeasts which include yeasts of the genera *Rhodotorula, Pichia, Candida*, *Metschnikowia*, *Debaryomyces, Zygosaccharomyces, Kluyveromyces, Kloeckera* (*Hanseniaspora uvarum*), and *Hansenula* (*anomala*), amongst others (Fleet et al., 1984; Henick-Kling et al., 1998; Pretorius et al., 1999; Lambrechts and Pretorius, 2000). Low levels of lactic acid bacteria (*Pediococcus* spp., *Leuconostoc mesenteroides*, and *Lactobacillus* spp.) are present in grape musts but their concentrations decrease dramatically with most of them dying off during alcoholic fermentation. Filamentous fungi found on grape skins include *Botrytis, Uncinula, Alternaria, Plasmopara, Aspergillus, Penicillium, Rhizopus, Oidium* and *Cladosporum* spp. but these also do not survive fermentation (Henick-Kling et al., 1998; Du Toit and Pretorius, 2000; Fleet, 2003).

Up to 15 different culturable yeast species are found on the skins of ripe grapes reaching numbers of up to $10^4$-$10^6$ cfu/g (Fleet, 2003; Jolly et al., 2003a). *Rhodotorula* spp., *Cryptococcus* spp, *Hanseniaspora/Kloeckera* spp. and *Metschnikowia/Candida* spp. are the

**Table 2.2** Anamorphs, teleomorphs and synonyms of some of the non-*Saccharomyces* yeasts in the Ascomycetous genera encountered in wine fermentations (Kurtzman & Fell, 1998).

| Anamorphic form | Teleomorphic form | Synonyms[1] |
|---|---|---|
| *Brettanomyces bruxellensis* | *Dekkera bruxellensis* | |
| *Candida colliculosa* | *Torulaspora delbrueckii* | *Saccharomyces rosei* |
| *Candida famata* | *Debaryomyces hansenii* | |
| *Candida globosa* | *Citeromyces matritensis* | |
| *Candida guilliermondii* | *Pichia guilliermondii* | |
| *Candida hellenica* | *Zygoascus hellenicus* | |
| *Candida lambica* | *Pichia fermentans* | |
| *Candida pelliculosa* | *Pichia anomala* | *Hansenula anomala* |
| *Candida pulcherrima* | *Metschnikowia pulcherrima* | *Torulopsis pulcherrima* |
| *Candida reukaufii* | *Metschnikowia reukaufii* | |
| *Candida sorbosa* | *Issatchenkia occidentalis* | |
| *Candida stellata* (now *Starmerella bombicola* or *Candida zemplinina*) | | *Torulopsis stellata* |
| *Candida valida* | *Pichia membranifaciens* | |
| *Kloeckera africana* | *Hanseniaspora vineae* | |
| *Kloeckera apiculata* | *Hanseniaspora uvarum* | |
| *Kloeckera apis* | *Hanseniaspora guilliermondii* | |
| *Kloeckera corticis* | *Hanseniaspora osmophila* | |
| *Kloeckera javanica* | *Hanseniaspora occidentalis* | |
| [3] | *Issatchenkia terricola* | *Pichia terricola* |
| [3] | *Kluyveromyces thermotolerans* (now *Lachancea thermotolerans*) | |
| [3] | *Saccharomyces kluyveri* | |
| [3] | *Saccharomycodes ludwigii* | |
| [3] | *Zygosaccharomyces bailii* | *Saccharomyces bailii* |
| [3] | *Pichia farinosa* | |

[1] Names sometimes found in older literature. [2] No teleomorphic form. [3] No anamorphic form.

most prevalent yeasts isolated from grape skins. *S. cerevisiae* are usually found on winery equipment and in fermenting must, and in much lower numbers on grape skins (Vaughan-Martini and Martini, 1995). Non-*Saccharomyces* species that have been isolated from winery equipment and cellar surfaces include *Candida* spp., *Cryptococcus* spp., *Pichia anomala, Pichia membranifaciens, Rhodotorula* spp., *K. apiculata, Metschnikowia pulcherrima* and *Debaryomyces hansenii* (Loureiro and Malfeito-Ferreira, 2003). Table 2.2 lists some of the non-*Saccharomyces* yeasts that have been isolated from wine environments.

Spontaneous fermentation is initiated by the non-*Saccharomyces* yeasts, in particular the apiculate lemon-shaped *Kloeckera apiculata* (teleomorph *Hanseniaspora uvarum)* yeasts (Martinand and Rietsch, 1891) which dominate the fermentation for the first 3-4 days. Species of *Rhodotorula*, *Candida*, *Pichia* and *Cryptococcus* are also present but in lower levels. The apiculate yeasts produce low amounts of ethanol (<4% v/v), and higher amounts of secondary compounds such as acetic acid (Müller-Thurgau, 1896). The non-*Saccharomyces* yeasts are sensitive to ethanol and start to die-off approximately three to four days into alcoholic fermentation as the concentration of ethanol increases, pH decreases and conditions in the fermenting must become more anaerobic (Fleet, 2003; Romano et al., 2003). Some are however able to survive and remain in the wine after alcoholic fermentation is completed (Fleet, 1984; Pardo et al., 1989) e.g. *C. stellata* (now *C. zemplinina*) and *Dekkera bruxellensis,* originating from cellar equipment. The elliptical, oval-shaped *S. cerevisiae*, known for its high ethanol tolerance, takes over from the non-*Saccharomyces* yeasts and completes the fermentation (Fleet, 1984; Romano et al., 2003). *S. cerevisiae* releases thermal energy during fermentation (William, 1982) causing a rise in fermentation temperature of up to 6˚ C (Goddard, 2008). It is believed that this temperature elevation plays an important role in the ability of *S. cerevisiae* to outcompete other yeasts: *S. cerevisiae* performs better at higher temperatures (>15˚C) (Arroyo-López et al., 2009) compared to other yeasts for example *Hanseniaspora uvarum*, *Torulaspora delbrueckii*, *Candida zemplinina*, *Pichia fermentans* and *Kluyveromyces marxianus* (Charoenchai et al., 1998; Salvadó et al., 2011a). Another advantage of *S. cerevisiae* is that it is able to respire the created ethanol in the presence of oxygen at a later stage of the fermentation (Piškur et al., 2006). *S. cerevisiae* constructs a niche for itself in the fermenting must via its metabolic activity which includes vigorous sugar metabolism, ethanol production and temperature elevation (Goddard, 2008; Salvadó et al., 2011b). *S. cerevisiae* has a high fermentation capacity and produces lower concentrations of secondary products. Figure 2.5 illustrates the concentrations and sequential succession of *Saccharomyces* and non-*Saccharomyces* yeasts during spontaneous wine fermentation. Wines produced by spontaneous fermentation are regarded as having improved complexity, mouth-feel and integration of flavours (Heard and Fleet, 1985).

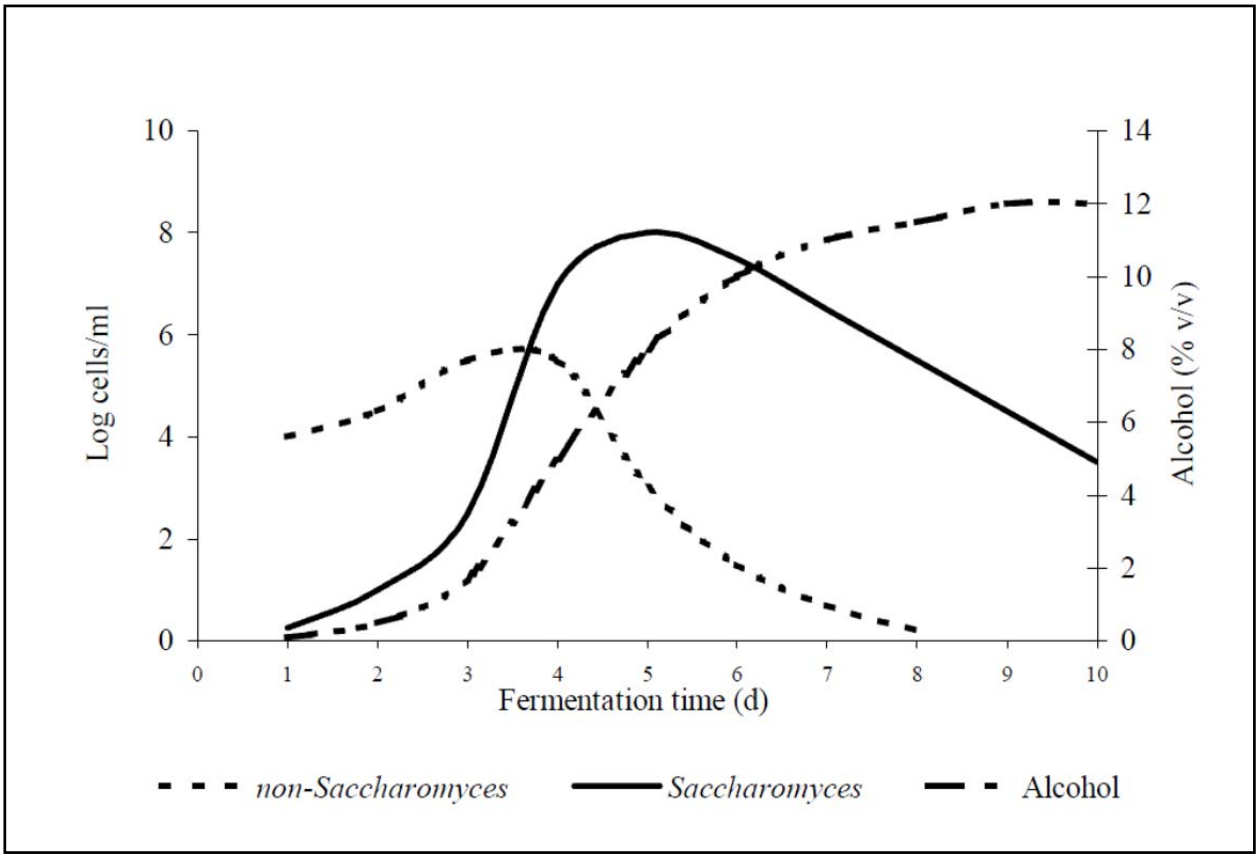


**Figure 2.5** Generalized growth of yeasts during spontaneous fermentation of wine (Dittrich and Grossmann, 2005).

When the aim is to deliver a particular wine style to a certain market segment, spontaneous fermentation may aid in producing distinctive wine styles that represent the yeast diversity of a specific region. Nevertheless, spontaneous fermentation poses a high risk of wine spoilage by undesired yeasts and bacteria, has a longer duration and the resultant wine can be highly unpredictable. In order to control fermentations and to obtain wines of reproducible and uniform quality, most winemakers inoculate their grape must with commercially available strains of *S. cerevisiae* to an initial concentration of $3x10^6$ cells/mL (Fleet, 2003; Bisson, 2004). This controlled fermentation process also leads to faster fermentations. *S. cerevisiae* out-competes other microbes for nutrients due to the high initial inoculum concentration, thereby granting greater microbial stability of the final wine product. *S. cerevisiae* dominates the fermentation and the influences of other microbes, especially the non-*Saccharomyces* yeasts, are so to speak eliminated. Certain non-*Saccharomyces* yeasts e.g. *Pichia* and *Hansenula* may produce off-odours such as acetic acid and acetaldehyde in wine (Du Toit and Pretorius, 2000). Selected *S. cerevisiae* strains are commercially available with known and desired genetic characteristics e.g. producing fruity flavours or low ethanol, giving winemakers greater control and predictability of the end-product (Jolly et al., 2006).

### 2.3.2 Growing interest in non-*Saccharomyces* wine yeasts

In the past, the influence of the non-*Saccharomyces* yeasts on wine was restricted and even eliminated by inoculation with pure *S. cerevisiae* cultures, because they were considered as undesired or spoilage yeasts (Amerine and Cruess, 1960). During spontaneous fermentation non-*Saccharomyces* yeasts can produce metabolites like esters, higher alcohols, acetic acid and acetoin that may result in a negative sensorial profile of the wine (Amerine and Cruess, 1960). However, in the past 3 decades great interest has grown in the potential beneficial role of non-*Saccharomyces* yeasts in wine biotechnology (Esteve-Zarzoso et al., 1998; Mendes Ferreira et al., 2001). It has been shown that some of the metabolites that these yeasts produce may be beneficial and contribute to the complexity of the wine when they are used in mixed fermentations with *S. cerevisiae* cultures (Zironi et al., 1993; Ciani and Maccarelli, 1998; Jolly et al., 2003; Romano et al., 2003; Mendoza et., 2007; Kim et al., 2008; Ciani et al., 2010; Mendoza and Farías, 2010; Rodríguez et al., 2010; Ciani and Comitini, 2011; Domizio et al., 2011). It is believed that when pure non-*Saccharomyces* yeasts are cultured with *S. cerevisiae* strains, their negative metabolic activities may not be expressed, or could be modified by the metabolic activities of the *S. cerevisiae* strains (Ciani and Comitini, 2011).

Various combinations of mixed and sequential fermentations of selected non-*Saccharomyces* yeasts with *S. cerevisiae* species have been investigated and have shed light on the positive albeit negative influence certain non-*Saccharomyces* yeasts may have on wine. Only some of the major findings will be highlighted here. *Candida stellata* (recently reclassified as Starmerella bombicola or as *C. zemplinina* depending on the strains) has a positive interaction with *S. cerevisiae* (Ciani and Ferraro, 1996; Ciani and Ferraro, 1998). It was shown to produce high amounts of glycerol with a production average of 11.76 g/l .The presence of *C. stellata* may improve the analytical profile of wine (Ciani and Maccarelli, 1998). In a substituted fermentation of *C. stellata* followed by inoculation with *S. cerevisiae*, decreased acetic acid and higher alcohols, and increased glycerol and succinic acid was observed (Ciani and Ferraro, 1998). *Torulaspora delbrueckii* is a good producer of succinic acid that contributes positively to the total acidity of wine with insufficient acidity and also produces ethanol to a concentration above 4% (v/v) (Ciani and Maccarelli, 1998). It contributes thus advantageously to the complexity of the wine aroma. Bely et al. (2008) reported that two *T. delbrueckii* strains produced low amounts of acetic acid in high sugar fermentations when used in mixed culture fermentations with *S. cerevisiae*, with a ratio of 20:1 *T. delbrueckii*/*S. cerevisiae*. The result was wine with 53% less volatile acidity and 60% less acetaldehyde than the wine inoculated with only *S. cerevisiae*. This co-inoculation leads to the improvement of the analytical profile of the sweet wine. *Candida pulcherrima* is a high producer of flavour enhancing esters and produced no undesirable volatiles during mixed fermentations with *S. cerevisiae* (Zohre and Erten, 2002; Jolly et al., 2003). Delayed fermentations were observed with sequential fermentations of *C. pulcherrima* followed by *S. cerevisiae* due to an antagonistic effect against *S. cerevisiae*

(Nguyen and Panon, 1998). The antagonistic effect was ascribed to the *C. pulcherrima* pigment produced by the C. *pulcherrima. K. apiculata* showed a large variability and significantly inverse correlations between either acetic acid and ethyl acetate formation and ethanol production, which could be profitably used for selection of yeast strains for industrial purposes (Ciani and Maccarelli, 1998). Mixed fermentations of *Pichia fermentans* with *S. cerevisiae* resulted in an increase in aromatic compounds such as acetaldehyde, ethyle acetate, 1-propanol, n-butanol, 1-hexanol, ethyl caprylate, 2,3-butanediol and glycerol (Clemente-Jimenez, 2005). During a mixed fermentation of *Lachancea thermotolerans* and *S. cerevisiae*, a significant decrease in acetic acid production was observed (Mora et al., 1990).

Certain non-*Saccharomyces* yeasts produce natural antimicrobial agents, called killer toxins, which may play important roles in the control of spoilage microflora in wine and other food products (Ciani and Comitini, 2011). After crushing the grapes winemakers add sulphur dioxide to their juice as antimicrobial agent and antioxidant to control the growth of undesirable oxidative microbes (Du Toit and Pretorius 2000). The concentration of sulphur dioxide in dry wines is approximately 160 g/L and can be up to 300-400 g/L for sweet wines. Sulphur dioxide negatively affects the respiratory systems of humans and animals and can damage vegetation. Killer toxins pose an alternative to the use of sulphur dioxide and other chemicals in order to control and prevent food spoilage (Heard and Fleet, 1987). *Tetrapisispora phaffi* produces a killer toxin, KpKt which can be used to combat the growth of apiculate yeasts present in the early stages of fermentation (Ciani and Fatichenti, 2001). The killer toxins of *Kluyveromyces wickerhamii* (Kwkt) and *Pichia anomala* (Pikt) are active against *Brettanomyces bruxellensis* species (Comitini et al., 2004).

Various non-*Saccharomyces* yeasts produce higher alcohols (n-propanol, isobutanol, isoamyl alcohol, active amyl alcohol) at lower levels than *S. cerevisiae* (Romano *et al*., 1993; Lambrechts & Pretorius, 2000) that can contribute to wine complexity.

### 2.3.3 Non-*Saccharomyces* yeasts with extracellular enzyme activity

Besides the attributes and features of non-*Saccharomyces* yeasts discussed above, quite a large number of them secrete a range of enzymes that may be of biotechnological use to the wine industry. This ability of these yeasts has drawn the interest of a number of researchers (Ogrydziak 1993; Charoenchai et al., 1997; Esteve-Zarzoso et al 1998; Dizy and Bisson, 2000; Fernandez et al., 2000; Van Rensburg and Pretorius, 2000; Strauss et al., 2001). Species that produce the greatest number of extracellular enzymes are *Starmerella bombicola*, *H. uvarum* (*K. apiculata*) and *M. pulcherrima* (*C. pulcherrima*). *S. cerevisiae*, the major wine yeast, is known as a poor producer of extracellular enzymes. The enzymes that non-*Saccharomyces* yeasts secrete include esterases, glycosidases, lipases, glucanases, pectinases, β-glucosidase, proteases and cellulases.

The enzyme β-glycosidase hydrolyses glycosidic precursors particularly from grapes to liberate e.g. odourous terpenes such as geraniol and linalool. Yeasts with extracellular β-glycosidase activity include *Debaryomyces* spp., *Candida* spp., *K. apiculata* and *P. anomala* and could potentially contribute to the production of different aromas to wine (Fernández et al. 2000; Mendes Ferreira et al., 2001; Fernández-Gonzáles et al., 2003; Rodriguez et al., 2004). *M. pulcherrima* and *Pichia membranaefaciens* strains demonstrated proteolytic activity against casein (Fernandez et al., 2000). Charoenchai et al. (1997) confirmed that some strains of *Candida* species and *K. apiculata* produced extracellular proteolytic or lipolytic activities. Strauss et al. (2001) screened 245 non-*Saccharomyces* isolates of oenological origin for the presence of 9 extracellular hydrolytic enzymes including proteases, pectinases, β-glucanases, lichenases, β-glucosidases cellulases, xylanases, amylases and sulphite reductase activity. Ten isolates of *C. stellata*, *C. pulcherrima*, and *K. apiculata* and one strain of *Debaryomyces hansenii* showed proteolytic activity. Nine isolates represented by *C. stellata*, *C. oleophila*, *C. pulcherrima*, *C. valida* and *K. apiculata* showed pectolytic activity. Isolates of *C. stellata*, *C. pulcherrima* and *K. apiculata* isolates showed lichenase activity. Cellulase activity was found in 11 isolates of *C. stellata*, *C. pulcherrima* and *K. apiculata.* Isolates with glucanase activity included *C. stellata, C. hellenica, K. apiculata, C. sorbosa, C. lambica, C. pulcherrima*. Yet again isolates of *C. stellata, C. pulcherrima* and *K. apiculata* had xylanase activity. It is clear that the extracellular enzymes produced by non-*Saccharomyces* yeasts hold strong potential in wine biotechnology. However, the screenings were performed mostly by plate assays and some liquid assays, and it is thus necessary to evaluate the extracellular hydrolytic activities of these yeasts in wine or wine simulating conditions to have a true reflection of the wine biotechnological applications that these enzymes hold.

The rest of this review will focus particularly on the proteases and how secretory aspartic proteases of non-*Saccharomyces* yeasts may be applied and contribute to the winemaking process.

## 2.4 The role of aspartic proteases in winemaking

### 2.4.1 Reduction in the risk of haze formation

One of the most important technical challenges for wine makers producing white wines is that of protein haze. This phenomenon occurs in white wine low in polyphenol content as a result of coagulation of proteins in the wine from unfavourable storage conditions, resulting in their aggregation. The denatured proteins can either precipitate to form an amorphous sediment or deposit, or can flocculate producing a suspended unstable and unsightly haze in bottled wine (Pocock and Waters, 2006). The presence of haze reduces the commercial value of the wine making it unacceptable for consumers as it may be perceived as microbial spoilage (Waters et al., 2005).

The total concentration of proteins in wines varies generally from 15 to 230 mg/L (Ferreira et al., 2002) and is mainly from the grapes but may also originate from yeast autolysis. The grape/wine proteins are essentially glycosylated (Moreno-Arribas et al., 2002). The addition of extrinsic enzymes e.g. pectinases to aid in juice extraction and lysozyme as antimicrobial agent adds slightly to protein content. The proteins responsible for haze formation originate mainly from the grape berries and have been classified as pathogenesis-related (PR) proteins although they are expressed in grape berries irrespective of pathogenic attack on the plant (Tattersall et al., 1997; Pocock et al., 2000). They are divided into two main classes, the thaumatin-like proteins (18 kDa-24 kDa) and the chitinases (30 kDa) (Waters et al., 1996; Van Sluyter 2009; Le Bourse et al., 2011). Recently however it has been suggested that the chitinases are mainly responsible for haze formation (Marangon et al., 2011). The PR proteins have low isoelectric point values (4.1 – 5.8) (Dawes et al., 1994), are acid-stable and are resistant to proteolytic hydrolysis. Their resistance to proteolysis is not because of inhibitors but due to their conformation and compact globular structure preventing proteases from having access to peptide bonds (Waters et al., 1992; Conterno and Delfini, 1993; Tattersall et al*.,* 2001). Non-protein factors such as polyphenols, the wine pH and the presence of polysaccharides in the wine also contribute to wine turbidity (Ferreira et al., 2002). Proteins are also important for foam formation and foam stability in sparkling wines. Approximately half of all wine proteins may be bound to grape phenolics (Somers and Ziemelis, 1973; Manteau et al., 2003).

Removal of protein haze thus forms an integral part of the white wine making process and has also enjoyed much attention as a research topic (Waters et al., 2005; Gonzalez-Ramos et al., 2008; De Bruijn et al., 2009; Marangon et al., 2011). Typically in industry, the haze caused by proteins is removed from the wine by bentonite fining. Bentonite, a cation exchanger, is a montmorillonite clay that carries a net negative charge and interacts electrostatically with and adsorbs to proteins, which carry a net positive charge at wine pH (Blade and Boulton, 1988; Ferreira et al*.,* 2002). The bentonite settles out from the wine, removing the proteins that are absorbed to it. The wine is then removed by clarification. Wines usually require approximately 1 g/l bentonite to remove haze and ensure stability; however different protein fractions may require distinct bentonite concentrations (Hsu et al., 1987; Dawes et al., 1994; Pocock et al*.,* 2003). Bentonite is non-specific and can bind to positively charged compounds in the wine other than proteins. Under certain conditions, bentonite fining may have an adverse effect on the quality of wine because some colour, flavour and aroma compounds may be removed together with the proteins (Voilley et al., 1990; Waters et al*.,* 2005). Another disadvantage of bentonite fining is that up to 20% of the wine volume can be lost as bentonite lees resulting in major economic losses for the wine industry (Lagace and Bisson, 1990).

Because of the drawbacks presented by bentonite fining, alternative treatments to remove haze-causing proteins have been investigated, amongst these the application of proteolytic enzymes (Bakalinsky and Boulton, 1985; Rosi et al., 1987). Extracellular proteolytic activities produced by *C. olea*, *C. lipolytica*, *Cryptococcus flavus*, *K. apiculata* and *C. pulcherrima* could be correlated with their ability to reduce wine haze (Lagace and Bisson, 1990). Dizy and Bisson (2000) demonstrated that strains of *Kloeckera* and *Hanseniaspora* produced the most proteolytic activity in grape juice, and affected the protein profile of the finished wines. Van Rensburg and Pretorius (2000) confirmed some degradation of wine proteins in wines incubated with proteases from *K. apiculata*. However, the haze forming potential of the wines was not significantly reduced by the protease activity produced (Dizy and Bisson, 2000). On the contrary, in some cases the haze level formed was greater in the fermentations with high proteolytic activity. Most commercial protease preparations have failed as they did not work under wine-making conditions (low pH and temperature) (Waters et al., 2005).

Pocock *et al.* (2003) demonstrated that the combined treatment of heat (90˚C for 1 minute or 45˚C for 24 h) and proteolysis (using Trenolin blank) reduced bentonite requirements significantly. Trenolin blank is a commercially available Aspergillopepsin enzyme with pectolytic and proteolytic activity. A 44% – 50% reduction in protein content was observed after heat and Trenolin blank treatment as well as reduced bentonite requirements and $SO_2$ content of the wine. No detrimental effect was observed on the organoleptic properties of the wine. Because of the compact and globular structure of thaumatin-like PR proteins, it has few exposed loops accessible to proteases causing them to be highly resistant to proteolysis and making heat treatment necessary for effective proteolysis (Tattersall et al*.,* 2001). Modification of winemaking procedures to induce protein unfolding should be investigated.

Besides proteolytic treatments, a range of alternatives to bentonite treatment have been investigated. These include ultrafiltration (Hsu et al., 1987) which could unfortunately also lead to losses in aroma compounds. The addition of certain polysaccharides has also been proposed. Arabinoglucans in wine derived from grapes and mannoproteins from fermenting yeasts reduce visible haziness by decreasing the particle size of the haze, known as the haze-protective factor (Waters et al., 1993). Mannoproteins are also released when wine is left on yeast lees, which stabilizes the wine against haziness. Mannoproteins comprised between 25% and 34% of the yeast cell walls (Nguyen et al., 1998). Mannoproteins released by *S. cerevisiae* are found in significant amounts in the wine. They have interesting oenological ability, e.g. inhibit tannin aggregation in wine, enhance the complexity and balance of aromas in wine (Bautista et al., 2007), and adsorb ochratoxin A (Caridi et al., 2006). Fining has been practiced with other proteins such as casein, albumin and gelatine which also reduce wine astringency (Ferreira et al., 2002). Flash pasteurization, using zirconium dioxide as alternative adsorbent (Marangon et al., 2011), and immobilised phenolic compounds have also been suggested

(Waters et al., 2005). Despite these investigations bentonite still remains the only industrially applied method for wine protein stabilization.

### 2.4.2 Increase in available assimilable nitrogen and wine aroma

Besides the potential to aid in haze reduction, the extracellular proteolytic activity of non-*Saccharomyces* yeasts of oenological origin may also hold potential to increase the assimilable nitrogen sources for the growth of microorganisms during fermentation.

Wine contains approximately 100 to 600 mg/L nitrogen mainly composed of peptides and free amino acids. Proteins may account for up to 2% of the total nitrogen content (Feuillat et al., 1998). The nitrogen-containing compounds in grape juice and wine are made up of an ammonia component and a more complex amino acid-based nitrogen component, e.g. amino acids, oligopeptides, polypeptides, proteins, amide nitrogen, bioamines, nucleic acids, amino sugar nitrogen, pyrazines, vitamins and nitrate (Henschke and Jiranek, 1993; Cramer et al., 2002). In wine, concentrations of these compounds are found in a broad range. Yeasts use a mechanism called nitrogen catabolite repression (NCR), which mediates the selection of good nitrogen sources by the expression of appropriate transport system (permeases) and the degradation of non appropriate permeases (Bell and Henschke, 2005). *S. cerevisiae*, the principal yeast used for fermentation, preferentially utilizes simple nitrogen sources such as ammonium ions and free alpha amino nitrogen compounds present in the form of primary amino acids such as glutamine and asparagine (Henschke and Jiranek, 1993). Arginine is quantitatively the most important amino acid utilizable by *Saccharomyces* in grapes and, subsequently unfermented juice. This amino acid is rapidly incorporated by the yeast at the start of fermentation and subsequently released back into the wine during autolysis. Secondary amino acids, such as proline and hydroxyproline are not metabolised to any great extent under winemaking conditions. Only low molecular weight peptides can also be utilized but grape proteins cannot be used as a source of nitrogen since *S. cerevisiae* lacks significant extracellular proteolytic enzymes to hydrolyse these proteins during fermentation. Proteinase A, a vacuolar protease of *S. cerevisiae,* is only secreted during autolysis following fermentation (Ogrydziak 1993; Alexandre et al., 2001). Recently however, Younes et al. (2011) identified proteolytic activity in *S. cerevisiae* PIR1 secreted during fermentation. Proteases secreted by non-*Saccharomyces* yeasts that are able to hydrolyse grape proteins under wine making conditions may increase the assimilable nitrogen sources by liberating peptides and possibly amino acids. On the other hand, certain by-products considered detrimental to health such as biogenic amines and ethyl carbamate can be produced by degradation of nitrogen compounds. Ethyl carbamate (urethane) is formed by the reaction of urea and ethanol. It is an undesirable compound of wine since it is considered as carcinogen and mutagen (Bell and Henschke, 2005).

An inadequacy of nitrogen-containing compounds of grape juices for wine fermentation has often been reported. Insufficient initial assimilable nitrogen sources, amongst other causes, may lead to stuck or sluggish fermentations. Sluggish or stuck fermentations, refers to those fermentations that commence normally but become slow or stop before must sugar concentrations are depleted (Henschke and Jiranek, 1993). Most winemakers therefore supplement their must with additional nitrogen sources such as diammonium phosphate (DAP) or ammonium sulphate (Hernandez-Orte et al., 2006). DAP added at the initial stage during the yeast growth phase increases the size of the yeast population, but has little effect on population size when added at later stages. Amino acid mixtures are also used to supplement grape must nitrogen concentrations. The greater efficiency of amino acid mixtures, especially balanced mixtures, compared with single nitrogen sources is linked to the ability of yeast to directly incorporate amino acids into protein, thereby minimising the need to maintain an energetically expensive amino acid synthetic capability (Bell and Henschke, 2005). A supplement of amino acids in grape juice could shorten fermentation time (Hermández-Orte et al., 2006). Utilization of nitrogen-containing compounds by yeasts is strain-dependent and the fermentation conditions also play a role (Valero et al., 1999), e.g. yeasts consume less nitrogen at low temperature and ethanol inhibits the uptake of most amino acids (Bisson, 1991).

Esters, higher alcohols, volatile fatty acids such as γ-butyrolactone, isobutanol and isobutyric acid and carbonyls are important contributors to the fermentation bouquet of wine (Fleet, 2003). These compounds principally arise as primary metabolites of yeast sugar and amino acid metabolism (Henschke and Jiranek 1993, Swiegers et al. 2005). Higher alcohols may be produced by catabolic transformation of branched-chain amino acids via the Ehrlich pathway. Therefore, the production of these flavour-active compounds during fermentation is influenced by the amino acid composition of the must. When concentrations of higher alcohols are low they contribute to the aroma complexity but an excess of higher alcohols has a negative impact on wine quality. These alcohols, together with organic acids, provide substrates for ester formation. Most esters confer pleasant flavours to wine, e.g. fruity and floral notes (Lambrechts and Pretorius 2000). Nitrogen compounds also regulate the formation of other volatiles, such as hydrogen sulfide, thiols/mercaptans and monoterpenes (Henschke and Jiranek, 1993). At high initial nitrogen content in must, the concentrations of total higher alcohols are at their lowest. Wines with high concentrations of esters, e.g. ethyl esters, and low higher alcohol concentrations, are associated with must with higher concentrations of amino acids (Hernández-Orte et al., 2006).

In summary, proteases can liberate peptides and amino acids contributing to the yeast's nitrogen pool required for coordinating amino acids, purine and pyrimidine synthesis (Bell and Henschke, 2005) needed for cell growth, flavour-active metabolites and also fermentation activity. However, nitrogenous compounds influence clarification and microbial stability.

Therefore careful nitrogen management is necessary as to control the growth of spoilage or undesired fungi and bacteria.

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# Chapter 3

# Research results

## Identification and partial characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384

# Identification and partial characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384


## Abstract

By using degenerate primers and Inverse-PCR, two extracellular aspartic protease encoding genes were identified and sequenced from two yeast species of oenological origin: *Metschnikowia pulcherrima* IWBT Y1123 named *MpAPr1* and *Candida apicola* IWBT Y1384 named *CaAPr1*. *MpAPr1* is 1137 bp long and the mature protein consists of 362 amino acids with a molecular weight of 39.2 kDa. The prepeptide had a predicted pI of 4.22 and one potential *N*-glycosylation site. The gene sequence of *MpAPr1* shared significant homology to only one nucleotide sequence, a hypothetical protein of *Clavispora lusitaniae* ATCC 42720 with 52% coverage and 65% identity scores. This is an indication of the novelty of the gene. The putative *CaAPr1* gene is 1101 bp long encoding a 367 amino acid long protein with a predicted molecular weight of 39.1 kDa and a pI of 4.33. It is thought that the putative protein follows a non-classical translocation process because no signal peptide could be predicted for the protein. Three potential *N*-glycosylation sites were predicted for the putative protein. Both MpAPr1 and CaAPr1 putative proteins showed homology to proteases of yeast genera. Heterologous expression of *MpAPr1* in *S. cerevisiae* YHUM272 confirmed that it encodes an aspartic protease. MpAPr1 production and secretion was shown to be induced in the presence of casein, grape juice proteins and to a lesser extent BSA. The *MpAPr1* gene was found to be present in 12 other *M. pulcherrima* strains; however plate assays revealed that the degree of protease activity was strain dependent.


**Keywords**: Extracellular aspartic proteases; *Metchnikowia pulcherrima*; *Candida apicola*; *MpAPr1*; *CaAPr1*; wine

**Abbreviations**: IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; YPD, yeast extract peptone dextrose; YNB, yeast nitrogen base; X-gal, 5-bromo-4-chloro-indolyl-galactopyranoside.

## 3.1 INTRODUCTION

Aspartic proteases [EC 3.4.23], also known as acid proteases (or APs), have been isolated from a range of organisms including retroviruses, bacteria, fungi, insects and vertebrates. Some common examples include pepsin, cathepsin D, chymosin, and the microbial penicillopepsin, with pepsin being the most studied AP. They are divided into three main families, A1 pepsin, A2 retropepsin, and A3 pararetroviruses. These enzymes may be intracellular or extracellular, they are active under acidic conditions, pH 2 – 5, have molecular weights ranging from 35 kDa to 50 kDa and isoelectric point (pI) values of 3.9 - 4.9 (Davies, 1990; Barrett et al., 1998; Rao et al., 1998). The APs have two reactive aspartic acid residues in their catalytic sites that are essential for their functioning. The two aspartic acid residues are found within two characteristic

hydrophobic sequences, Asp32-Thr-Gly-Ser in the N-terminal domain, and a corresponding Asp215-Thr-Gly-Ser/Thr in the C-terminal domain (according to pepsin numbering) (Dunn, 2002). The enzymes are inhibited by pepstatin A, a hexapeptide from *Streptomyces* (Davies, 1990) and are also sensitive to diazoacetylnorleucinemethyl (DAN), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) in the presence of copper ions (http:/merops.sanger.ac.uk).

These proteases are monomers and have a bilobal structure with the active site cleft located at the interface where the two lobes meet. Each lobe contributes an aspartic acid residue to the active site. The enzymes also have a flap region that closes over the active site cleft. The retroviral APs are smaller homodimers formed by two identical domains (Tyndall et al., 2005). APs are endopeptidases that cleave peptides of at least 6 residues long with hydrophobic residues in the scissile bond. The mechanism of action is a general acid-general base mechanism where one of the aspartic acid residues in the active site act as an acid, while the other function as a base. A water molecule, hydrogen bonded between the two aspartic acid residues, plays a central role in the catalytic reaction (Dunn, 2002).

The extracellular acid proteases of fungi and yeast have been studied extensively, for example those of *Aspergillus*, *Penicillium* and *Candida* spp. They show specificity against aromatic or bulky amino acid residues on both sides of the scissile peptide bond, and their action is less stringent than that of e.g. pepsin (Ogrydziak, 1993; Rao et al., 1998). The pathogenic yeast, *Candida albicans* has 10 secreted aspartic proteases (Saps), which are encoded by 10 genes (*SAP1 – SAP10*) (Naglik et al., 2004). The secretory pathway of the APs secreted by *C. albicans* and other *Candida* spp. has been investigated. The process is similar to that found in *Saccharomyces cerevisiae*. The *SAP* genes are translated as pre-pro-enzymes on the rough endoplasmic reticulum (ER). The pre-peptide (signal peptide) is cleaved in the (ER) by a signal peptidase complex followed by glycosylation and formation of disulfide bonds. The Saps undergo further maturation during transportation via the Golgi apparatus, where processing is performed by a Kex2 protease (Togni et al., 1996; Newport and Agabian, 1997; Naglik et al., 2004). At the end of the secretory pathway, Saps are either incorporated to the cell wall via a GPI anchor or released into the extracellular space (Albrecht et al., 2006).

Most commercial winemakers inoculate their grape juice with commercially available *Saccharomyces cerevisiae* strains (inoculated at an initial concentration of $3x10^6$ cells/ml) in order to obtain a uniform and predictable product, and also to ‘minimize’ the growth of other microbes present in the grape must (Fleet, 2003). Recently however, the role and contribution of the non-*Saccharomyces* yeasts (mostly present during the initial stages of spontaneous wine fermentation) in the final wine product have been investigated (Lambrechts and Pretorius, 2000; Jolly et al., 2006; Ciani and Comitini, 2011). These include yeasts from the genera *Rhodotorula*, *Pichia*, *Candida*, *Metschnikowia, Kloeckera* and *Hansenula,* amongst others. Some of the non-

*Saccharomyces* yeasts produce metabolites like esters, higher alcohols, acetic acid and acetoin that may contribute positively or negatively to the flavour complexity of the wine (Ciani and Maccarelli, 1998; Mendoza and Farías, 2010). They also secrete enzymes, e.g. pectinases, β-glucosidases and proteases that might be of interest to the wine maker. The proteases cannot only increase the concentration of assimilable nitrogen sources for the growth of desirable (and spoilage) microbes, but also improve clarification and possibly reduce wine protein haze (Lagace and Bisson, 1990; Pocock et al., 2003; Jolly et al., 2006). Treatments such as bentonite fining and ageing on total yeast lees to reduce the risk of haze formation can be expensive, amongst other disadvantages (Waters et al., 2005). Successful protease treatment prior to fining may reduce costs. Although many non-*Saccharomyces* wine yeasts have been shown to have extracellular proteolytic activity, the characteristics of these enzymes have not been studied at genetic level. This study describes the isolation and characterization of acid protease-encoding genes from two wine associated yeasts, *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. The deduced protein sequences were characterized by *in silico* investigations and the gene isolated from *M. pulcherrima* IWBT Y1123 was expressed in *S. cerevisiae* YHUM272. Protease induction studies were also performed.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Strains, plasmids and culture conditions

The strains used in this study as well as their sources are listed in Table 3.1. *Yarrowia lipolytica* UOFS Y1698 was provided by Prof. Lodewyk Kock, University of the Free State, South Africa. *Metschnikowia pulcherrima* FOEB L0642 was provided by Prof. Isabelle Masneuf-Pomarède, ENITA de Bordeaux, France. The yeast strains were maintained on YPD agar (Biolab diagnostics, Wadenville, South Africa) and freshly cultured prior to use in experiments. Yeast strains were grown at 30°C in YPD broth (Biolab diagnostics). Plasmids were constructed and grown in *Escherichia coli* DH5α grown at 37°C on a rotary shaker at 150 rpm in Luria–Bertani medium (Biolab diagnostics, Wadenville, South Africa), supplemented with 100 mg/l ampicillin (Ampicillin sodium salt, Sigma-Aldrich, Johannesburg, South Africa), 0.5 mM IPTG (Sigma-Aldrich) and 80 µg/ml X-gal (Sigma-Aldrich) when appropriate. Chemically competent *E. coli* DH5α cells were transformed according to the Promega Technical Manual (TM042, Promega, Whitehead Scientific, Cape Town, South Africa). Solid media contained 20 g/l agar. All strains were stored at −80°C in broth containing 40% (v/v) glycerol. All plasmids used in this study are listed in Table 3.2.

*Induction of protease secretion*. To induce protease secretion, the method of Lagace and Bisson (1990) was followed with modifications. The yeast strain *M. pulcherrima* IWBT Y1123 was grown in 10 ml MYGP medium (0.3% malt, 0.3% yeast extract, 0.5% peptone and 2% (w/v)

glucose) for 24h at 30°C on a rotating wheel. The cells were harvested by centrifugation at 5000 rpm for 5 minutes at 4°C and washed twice with 0.9% physiological water. The cells were then added to a base medium containing 1% glucose, 0.1% Difco YNB (without amino acids and ammonium sulphate), and 0.066% ammonium sulphate. The pH was adjusted to 5.5 with 1N HCl and the medium was filter sterilized. The culture was grown for 24h at 30°C with shaking at 160 rpm. After the 24h incubation period, the cultures were spiked with different nitrogen sources which included 0.250 mg/ml grape juice proteins, 0.250 mg/ml bovine serum albumin (Fraction V, Roche, Mannheim Germany), 0.250 mg/ml casein (Sigma-Aldrich), and 0.250 mg/ml ammonium sulphate (Merck, Wadeville, South Africa). As negative control, one flask was not spiked with any nitrogen source. The cultures were grown for a further 48h at 30°C and 160 rpm. At the end of induction the cultures were centrifuged at 5000 rpm for 10 minutes at 4°C. The cell pellet was frozen at –80°C for RNA extraction. The crude supernatant was filtered through a 0.45 µm filter and concentrated 6 times by filtration using Amicon centrifugal filter units (Millipore, Davies Diagnostics, Randburg, South Africa) with a 10 kDa cut-off. The retentate was used as the crude protease preparation and stored at 4°C. Total protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Labs., Hercules, CA, USA) according to the manufacturer's protocol. Experiments were performed in triplicate.

Grape juice proteins were extracted from Chardonnay grape juice by acetone precipitation. Grape juice was obtained from the Nietvoorbij Experimental cellar (Agricultural Research Council, Stellenbosch, South Africa) from grapes harvested during the 2011 harvest season. One volume of ice cold 100% acetone (Merck, Wadeville, South Africa) was added to the grape juice and incubated overnight at −20°C. The proteins were recovered by centrifugation at 10000 rpm for 30 minutes at 4˚C. The protein pellet was washed twice with 4:1 acetone to water and dried overnight at −20°C. The proteins were re-suspended in 0.05 M citrate phosphate buffer, pH 3.5.

*Acid protease activity determination*: Acid protease activity was determined by spotting of the cells on skim milk plates at pH 3.5 according to Charoenchai et al. (1997). Pure colonies were suspended in 10 µl of mQ water (Millipore) and spotted on plates. Plates were incubated at 30˚C for 3 days. Enzymatic activity was visualised by a zone of clearance of at least 1 mm around the edges of the yeast colony. All assays were performed in triplicate.

### 3.2.2 Molecular biology and Bioinformatics techniques

#### 3.2.2.1 Nucleic acid extraction

Genomic DNA was isolated from 24h YPD cultures grown at 30˚C with shaking at 160 rpm. Genomic DNA and RNA were extracted from yeast cultures using the methods described in Current Protocols in Molecular Biology (2008) according to Hoffman and Winston (1987) and

Collart and Oliviero (1993), respectively. Plasmid DNA was recovered from *E. coli* cultures using the QIAprep Spin Miniprep Kit (Qiagen, Southern CrossBiotechnology, Cape Town, South Africa) according to the manufacturer's instructions. Genomic DNA and RNA concentrations were quantified using the NanoDrop® ND-1000 Spectrophotometer (Wilmington, USA).

#### 3.2.2.2 *In silico* analyses

Homology searches of nucleotide sequences *and database searches* were carried out using the Basic Local Alignment Search Tool (BLAST) service provided by the National Centre for Biotechnology Information (NCBI) website *(*www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments and comparisons of DNA sequences were performed using the programs of MultAlin (http://multalin.toulouse.inra.fr/multalin/*) and* ClustalW alignment software provided by the European Biotechnology Institute (EBI) (www.ebi.ac.uk/clustalw). The nucleotide sequences were translated into amino acid sequences and alignments were performed using Transeq and Showalign from the EMBOSS software suite (http://www.ebi.ac.uk) (Rice et al., 2000). Degenerate primers were designed using the following program http://hcgs.unh.edu/protocol/basic/pcrdegenpri.html. The secretion signal peptide of the putative protein was detected by the use of the software (http://cbs.dtu.dk/services/SignalP/) (Version 3.0) on the CBS website. For the calculation of the pI and molecular weight of the putative proteins the software of the Expasy website was utilised (http://web.expasy.org) and for prediction of N-glycosylation sites, http://www.cbs.dtu.dk/services/NetNGlyc/. To investigate and compare the conserved regions and motifs in the protein sequences with other proteins the conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) at NCBI was used.

#### 3.2.2.3 PCR methods

The primers and PCR programmes used in this study are listed in Table 3.3 and Table 3.4, respectively. Primer oligonucleotide sequences were synthesised by Integrated DNA Technologies (IDT, Whitehead Scientific). All PCRs were performed using the Phusion High-Fidelity DNA Polymerase System (Finnzymes, Vantaa, Finland) unless otherwise specified. PCR programmes were run with an Applied Biosystems 2720 Thermal Cycler (California, USA). In order to generate sticky ends, PCR products were incubated for a further 10 min at 72˚C with addition of dATP nucleotides (Takara Bio Inc., Separations, Randburg, South Africa), when necessary. Fragments were purified from agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, California, USA). PCR products were cloned with pGEM®-T Easy Vector Systems (Promega).

*Yeast identification*: In order to identify the yeasts, gDNA were amplified with the primers ITS1 and ITS4 (Table 3.3) using the PCR programme (Table 3.4) previously described (White et al., 1990; Lott et al., 1998).

*Degenerate PCR.* To obtain the partial gene sequences of the acid proteases of *M. pulcherrima* IWBT Y1123, *A. pullulans* IWBT Y1008 and *C. apicola* IWBT Y2384 a PCR based approach was followed. The amino acid sequence of the aspartic protease of *Y. lipolytica* CLIB122 strain (Accession number XP_504725.1; Dujon et al., 2004) was BLASTed and aligned with aspartic proteases of other yeast species. The conserved sequences, which are also identified as the active site regions of the enzymes, were used to design degenerate primers. An alignment of the acid protease of CLIB122 with those of three other yeasts is shown in Figure 3.1.

*Inverse PCR (IPCR).* Genomic DNA (gDNA) was digested separately with 5 different restriction enzymes, *Eco*RI, *Eco*RV, *Dra*I, *Hpa*I and *Xba*I (Roche Diagnostics, Randburg, South Africa). These enzymes were selected based on (a) they do not cut within the sequence obtained from amplification with degenerate primers and (b) would result in fragments between 2000 and 4000 bps. This was determined by using the genome sequence of *Candida glabrata* (Koszul et al., 2002), which is closely related to *M. pulcherrima*. In short, 200 ng genomic DNA was digested in 50 µl reaction mix for 2h at 37˚C followed by inactivation of the restriction enzymes at 65˚C for 20 minutes. Self-ligation proceeded in 200 µl reactions at 16˚C overnight using T4 ligase (Promega). The IPCR was performed with 2 µl of the ligation reaction mix in 50 µl reactions using Elongase (Invitrogen, USA). After amplification, the reaction mix was diluted 100 times and 2 µl thereof was used as template for a nested IPCR. The fragments were cloned and sequenced as previously described. After *in silico* analysis, new primers were designed based on the sequences obtained from the IPCR. These were used to amplify the full length genes encoding the acid proteases from gDNA.

*Reverse transcription-PCR.* Reverse transcription was performed using the ImPromII™ Reverse Transcription System of Promega according to the manufacturer's instructions. The Oligo$(dT)_{15}$ primer of the kit was used to initiate reverse transcription of poly$(A)^+$ mRNA molecules. PCR on the obtained cDNA was carried out as described above. cDNA was either amplified with the *MpAPr1* encoding primers (MpAPr1-F/MpAPr1-R) or the NL4/NL1 primer set encoding the constitutively expressed 26S rDNA gene (Kurtzman and Robnett, 1998).

### 3.2.3 Cloning and heterologous expression in *S. cerevisiae* YHUM272

After amplification of the protease encoding gene from gDNA, the PCR product was cloned into pJET1.2 CloneJet™PCR Cloning Kit (Fermentas) followed by transformation of *E. coli* DH5α. After plasmid extraction, the gene was excised from the pJET1.2 plasmid by restriction digestion with *Bam*HI and *Xho*I (Roche Diagnostics) and ligated into the corresponding sites of the pCEL13 expression vector using T4 DNA ligase (Fermentas) and transformed into *S. cerevisiae* YHUM272. Yeasts were transformed using an electroporation method by Volschenk et al. (2004). Positive transformants were selected on minimal medium (0.17% Difco

YNB without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 2% glucose) plates supplemented with 40 µg/ml tryptophan, 60 µg/ml leucine and 20 µg/ml histidine.

To confirm successful transformation of the yeast with pCEL13 containing the gene, colony PCR with positive transformants as template, were performed. The primers 5′-KPNPGK-631, which recognizes the promoter sequence on the plasmid, and Mpulch_IPCR_R2 (nested) were used.

### 3.2.4 DNA sequencing

DNA strands were sequenced in an ABI 3130XL Genetic Analyzer at the Central Analytical Facility (Stellenbosch University) using the SP6 and T7 primers (Promega) or pJET1.2 specific primers (Fermentas, Inqaba Biotec, South Africa).

### 3.2.5 Protein work

#### 3.2.5.1 SDS-PAGE and zymography

SDS-PAGE analysis was performed on concentrated culture supernatants as previously described by Laemmli (1970) with 12.5% bis-acrylamide gel on a Bio-Rad Mini-PROTEAN® Tetra Cell System (Bio-Rad Labs., Hercules, CA, USA). Zymography was performed according to Folio et al. (2008) at pH 3.5 and using gelatine as co-polymerized substrate. To visualize protein bands, gels were stained with Coomassie blue R-250.

#### 3.2.5.2 Protein sequencing

Selected protein bands were excised from bis-acrylamide gels and, following trypsin digestion, were sequenced by Nano-LC and LC-MS/MS at the Central Analytical Facility of Stellenbosch University (Bellville, South Africa). Experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electropsray source. Thermo Proteome Discoverer 1.2.0.208 (Thermo Scientific, Bremen, Germany) were used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra against the deduced protein sequence using a decoy database search with FDR 0.01. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 2 tryptic peptides per proteins, a Mascot score of more that $p<0.05$ and peptides of high and medium confidence as determined by Proteome Discoverer with a false discovery rate of 0.01.

## 3.3 RESULTS

### 3.3.1 Protease activity screening and strain selection

A collection of 308 yeast strains were isolated from grape juice which was pressed from Chardonnay grapes harvested during the 2009 harvest season in Stellenbosch, South Africa. The strains were screened for extracellular protease activity by performing plate assays under acidic conditions (pH 3.5). Three strains displayed strong protease activity at pH 3.5 namely IWBT Y1123, IWBT Y1008 and IWBT Y1384 (data not shown). These isolates were identified to the species level by PCR amplification of the internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA gene regions, sequencing of the amplicons and performing BLAST searches. The strains were named *Metschnikowia pulcherrima* for IWBT Y1123, *Aureobasidium pullulans* for IWBT Y1008 and *Candida apicola* for IWBT Y1384, according to closest related species with identity scores of more than 98%.

### 3.3.2 Isolation and cloning of the aspartic protease encoding genes

Degenerate primers were designed based on amino acid sequence similarities (the conserved active site regions) of aspartic proteases from various yeast species, mainly *Y. lipolytica* and some *Candida* spp. PCR was performed on gDNA of *Y. lipolytica* UOFS-Y1698, used as reference strain, *A. pullulans* IWBT Y1008, *M. pulcherrima* IWBT Y1123 and FOEB L0642 (of South African and French origin respectively), and *C. apicola* IWBT Y1384. The amplicons generated by PCR were run on an agarose gel and the fragments corresponding to the expected size (~570 bp based on the length of the sequence between the active site encoding regions of the *Y. lipolytica* CLIB122 gene) were excised and used as template for a second amplification with the same primers (Figure 3.2). The fragments were excised from the agarose gel, ligated into the pGEM®-T Easy vector and transformed into *E. coli* DH5α. After extraction the fragments in the plasmids were sequenced. Homology searches were performed with the deduced protein sequences of the PCR fragments. The deduced sequences of *M. pulcherrima* IWBT Y1123 and *C. apicola* IWBT Y1384 yielded hits with known aspartic proteases. However, that of *A. pullulans* IWBT Y1008 displayed no homology to acid proteases. Experiments were thus continued with the sequences of the two positive strains. New primers specific for each strain were designed from the putative partial gene sequences to perform nested-IPCR as described in the Materials and Methods section. The PCR products from the nested-IPCR are illustrated in Figure 3.3. The fragments obtained from amplification with the nested primers from the digestion with *Eco*RI*, Dra*I, *Eco*RV and *Hpa*I for IWBT Y1123, and *Eco*RI*, Dra*I, *Eco*RV and *Xba*I for IWBT Y1384 were cloned and sequenced as before followed by performance of BLAST searches of the deduced amino acid sequences in order to confirm acid protease homology. Positive hits were obtained. Finally, primers were designed to amplify the full length genes encoding putative aspartic proteases (Figure 3.4). According to the sequences obtained from these new primers the putative gene of *M. pulcherrima* IWBT Y1123 is 1137 bp long and that of

*C. apicola* IWBT Y1384 is 1101 bp long. The nucleotide and their deduced amino acid sequences were analysed *in silico*.

### 3.3.3 *In silico* analysis of the putative gene and deduced protein sequences

Alignments of the sequences obtained, nucleotide and amino acid, are illustrated in Figures 3.5 and 3.7. Specific features and different characteristics of the sequences are listed in Table 3.5. The putative genes were named *MpAPr1* from the *M. pulcherrima* strain and *CaAPr1* from the *C. apicola* strain, respectively. The length of the putative genes differ only by 36 bp with the *MpAPr1* being 1137 bp long and *CaAPr1* being 1101 bp long. The molecular weight of the putative preproenzymes deduced from the gene sequences are 40.9 kDa (*MpAPr1*) and 39.1 kDa (*CaAPr1*). The predicted secretion signal peptide cleavage site for the preproprotein of *M. pulcherrima* IWBT Y1123 is between $Gly^{16}$ and $Met^{17}$ with the first 16 amino acids being the secretion signal peptide. Conflicting results were obtained from different on-line databases and software programmes with regard to the prediction of the signal peptide of the enzyme of *CaAPr1*. The protein was found not to have a signal peptide, but was still considered to be a secreted protein that follows a non-classical secretion pathway according to the CBS Secretome 2.0 Server, http://www.cbs.dtu.dk/services/SecretomeP/. Only one potential *N*-glycosylation site could be identified for the *MpAPr1* protein while three potential sites were predicted for the *CaAPr1* protein. Both putative proteins were classified as eukaryotic aspartic proteases (A1 family, EC3.4.23) according to the conserved catalytic motifs (DTGS/DSGT) and active site flap regions, which are typically conserved motifs of this family of proteases. Figures 3.6 and 3.8 illustrate the major conserved domains of aspartic proteases identified in the putative proteins, which includes the catalytic residue motifs, inhibitor binding sites, catalytic motifs and the active site flap regions.

### 3.3.4 Putative identification based on homology studies

The putative gene sequence of *MpAPr1* was compared to other nucleotide sequences by performing homology searches against the NCBI database. Only one significant hit was observed; *Clavispora lusitaniae* ATCC 42720 hypothetical protein, mRNA (Accession number XM_002615870.1) with 52% coverage and 65% identity. This is an indication of the novelty of the gene sequence. The rest of the hits shared between 86% - 96% identity, but only 2% - 3% coverage with the gene sequence. These included identity scores with short coding regions of *Dermanyssus gallinae* (red mite) (HE565350.1), *Aspergillus terreus* (XM_001211527.1), the plant pathogen *Albugo laibachii* (FR824182.1), *Drosophila mojavensis* (fruit fly) (XM_002004513.1), *Yarrowia lipolytica* (XM_500144.1) and even *Homo sapiens* (AC106053.6). The hits showed coverage of the gene sequences that encode one of the active site regions of the putative acid protease indicating the potential acid protease nature of *MpAPr1*.

Seven of the proteins in the NCBI database that share similarities with the putative protein of *MpAPr1* are listed in Table 3.6. The best score was with the aspartyl protease of *Clavispora lusitaniae* ATCC 42720, the same as with the nucleotide alignment. Unlike with the nucleotide sequences, all the matches are to fungal proteins, particularly of the *Candida* genus with much higher coverage and identity scores.

Similar results were obtained for the gene sequence of *CaAPr1*. Very low coverage scores (between 2% and 7%) with relatively high identity scores (80% to 90%) were observed for the region corresponding to the active site encoding gene sequences. The best score was given with the aspartyl proteinase (PAPA) gene of the soil fungus *Trichoderma asperellum* (AY611632.1). Although most of the matches were of fungal species, others included aspartic peptidases and unidentified proteins from a variety of species such as *Ajellomyces dermatitidis* mRNA prepropenicillopepsin XM_002625219.1, the house mouse *Mus musculus* (JN950245.1) and the fish *Dicentrarchus labrax* (AM943112.1). Again, it is clear that the active site encoding regions are highly conserved throughout different species. Table 3.7 shows seven of the proteins that share similarities with the putative protein of *CaAPr1*. All the proteins are from fungal species.

A phylogenetic tree of the two putative proteins with the proteins listed in Tables 3.6 and 3.7 is shown in Figure 3.9. It is clear that the CaAPr1 protein is more closely related to the aspartic proteases of *Y. lipolytica* than the MpAPr1 protein is. The MpAPr1 protein on the other hand, is closely related to the *Candida* spp.

The role of *M. pulcherrima* strains has been investigated in fermentation studies (Jolly et al., 2003 and Rodríguez et al., 2010) and is known to secrete a number of enzymes (Charoenchai et al., 1997; Strauss et al., 2001 and Jolly et al., 2006), and therefore holds great biotechnological potential, especially for the wine industry. However, the proteolytic enzyme(s) of *M. pulcherrima* has not been studied at a genetic level. It was subsequently decided to continue studies only with the putative *MpAPr1* gene.

### 3.3.5 Heterologous expression of the protease encoding gene of *M. pulcherrima* IWBT Y1123 in *S. cerevisiae* YHUM272

The *MpAPr1* putative gene sequence of *M. pulcherrima* IWBT Y1123 was cloned into the shuttle vector pCEL13 for expression in the laboratory strain *S. cerevisiae* YHUM272. The yeast was also transformed with the vector not containing the gene. Successful transformation was confirmed by PCR with primers 5′-KPNPGK-631 and Mpulch_ IPCR_ R2 (nested). Colony PCR was performed on the yeast transformed with *MpAPr1*, the yeast transformed with the empty vector as well as with the untransformed yeast. The presence of the putative gene fragment

was detected only in the yeast transformed with *MpAPr1* and not in the other strains (Figure 3.10A).

The extracellular acid protease activity of the transformed strains was investigated by plate assays. Only the recombinant strain and the IWBT Y1123 strain showed extracellular protease activity as indicated by the zone of clearance around the colonies (Figure 3.10B). No activity was observed from the strain that contained the empty plasmid or from the untransformed yeast. The results confirmed that the *MpAPr1* gene from *M. pulcherrima* IWBT Y1123 indeed encodes an extracellular acid protease enzyme.

### 3.3.6 Induction and substrate specificity investigation

The induction and substrate specificity of the *MpAPr1* gene of *M. pulcherrima* IWBT Y1123 were studied upon exposure to different nitrogen sources by simultaneously investigating gene expression and the presence of the protease in the extracellular medium. After pre-culturing *M. pulcherrima* IWBT Y1123, cells were transferred to minimal medium and incubated for 1 day, after which cultures were spiked with different nitrogen sources and incubated for 2 more days, as explained in Material and Methods. The different nitrogen sources included ammonium sulphate, BSA, casein and grape juice proteins. As a control, a fifth culture received no nitrogen source addition for the final two-day incubation. The experiments were performed in triplicate. The cells were harvested by centrifugation and the culture supernatants were concentrated by ultra-filtration and used as the crude protease preparations. Total RNA was extracted from the harvested cells. Reverse transcription was performed on mRNA and cDNA was used as template for PCR. The 28S rDNA gene was used as constitutively expressed (housekeeping) gene and was present in all the samples. The amplification showed a similar expression level of the housekeeping gene in all the samples (Figure 3.11). Thus the amount of *MpAPr1* transcript could be compared between the cultures containing different nitrogen sources. The results indicated that expression was induced in the presence of casein and grape juice proteins and only slightly in the presence of BSA. No amplicon, and thus no expression, was observed either in the presence of ammonium sulphate or in the absence of any nitrogen source.

In order to assess the presence of the protease and its activity in the extracellular medium, the concentrated culture supernatants representing total extracellular proteins were analysed by SDS-PAGE. The protease activity was visualized by zymography at pH 3.5 using gelatine as protease substrate. Figure 3.12 shows the extracellular proteins of *M. pulcherrima* IWBT Y1123 grown in the presence of casein. Lane (a) shows the profile of a commercially available casein preparation alone before incubation with *M. pulcherrima* IWBT Y1123. The bands between 35 kDa and 25 kDa correspond to the molecular weights of two of the four subunits of casein. Lane (b) shows the total extracellular proteins of the culture supernatant after incubation with casein. The casein bands were still clearly visible but another band between 35 kDa and 40 kDa (at approximately 39 kDa) was also visible. This band was excised from the gel and sequenced.

After 3 days of incubation in the medium without the presence of the yeast, no degradation was observed of the casein (lane c). No protease activity was observed in the zymogram.

The analysis of the extracellular proteins of *M. pulcherrima* IWBT Y1123 after incubation with BSA as nitrogen source is shown in Figure 3.13. The first blank sample, lane (a), shows the profile of BSA alone before incubation with *M. pulcherrima* IWBT Y1123. The band at approximately 68 kDa corresponds to the molecular weight of BSA. The profile in lane (b) illustrates the proteins in the culture supernatant after incubation with *M. pulcherrima* IWBT Y1123. An evident degradation of BSA is observed by a decrease in intensity of the band at 68 kDa. A number of low molecular weight bands are present which may be a combination of hydrolysis artefacts of BSA and proteins secreted by the yeast. Lane (c) shows the profile of BSA after 3 days incubation without the yeast. No degradation of BSA was observed indicating that the protein is stable under the incubation conditions and hydrolysis must therefore be as a result of proteolysis. No proteolytic activity could be observed by zymography either in the BSA or the blank sample.

The analysis of the extracellular proteins of *M. pulcherrima* IWBT Y1123 grown on grape juice proteins is illustrated in Figure 3.14. Lane (a) shows the profile of the grape proteins after extraction from grape juice by acetone precipitation. The band at ~60 kDa may be grape invertase (Marangon et al., 2009 and Le Bourse et al., 2011) and the intense band just beneath 25 kDa may be the common grape proteins, thaumatin-like (22 kDa) and/or chitinase (25 kDa) (Waters et al., 1992 and Waters et al., 1996). These proteins are known to be very stable due to their conformation thus degradation due to incubation conditions was not expected (Waters et al., 2005). Lane (b) shows the extracellular protein profile of *M. pulcherrima* IWBT Y1123 grown in the presence of grape juice proteins. The band at ~35 kDa disappeared and those at 10 kDa, 25 kDa and 60 kDa were fainter. New faint bands could be seen between 25 kDa and 40 kDa. The arrow points to the band that was excised from the gel and sequenced. In lane (c) very faint bands could be seen and a slightly more intense band was visible for the culture grown on ammonium sulphate. Faint bands were also seen for the blank sample where no nitrogen source was added. These could be proteins secreted by the yeast or released from early autolysis due to starvation of the yeast. Activity could be visualized for the culture supernatant grown with grape juice proteins but not with ammonium sulphate or the blank sample. This indicated the presence of the protease. A clear zone was visible at the top of the lane and another clear zone was observed at just above 40 kDa making it hard to estimate the molecular weight of the protease from the zymogram activity.

In order to identify whether the protease was indeed present in the culture supernatants, protein bands corresponding to the expected size as predicted by prediction software were manually excised from lanes in SDS-PAGE gels from casein-induced and grape protein-induced protein profiles. The bands were trypsin digested and Nano-LC-MS/MS analysis was performed. The

obtained peptide sequences were processed against the MASCOT database and the deduced amino acid sequence of the *MpAPr1* gene. The protein bands were positively identified to the deduced protein sequence both from casein-induced and grape protein-induced cut-outs. For the band from casein-induced culture identification of the protein resulted in 21.27% sequence coverage of the protein with 6 peptides identified by the MASCOT search engine, and for the band from the grape protein-induced, culture identification resulted in 19.9% sequence coverage with 5 peptides identified.

A positive correlation was therefore found between the expression of the gene at transcription level and protein production by the presence of the protease in the extracellular matrix of the yeast grown in casein and grape juice proteins. However, activity could not be visualized in the zymogram with casein. This could be because the concentration of the protease in the culture supernatant was too low.

### 3.3.7 Genetic screening of 12 *M. pulcherrima* strains for the presence of *MpAPr1*

The identity of twelve *M. pulcherrima* strains previously isolated from grape juice of different harvest seasons was confirmed by amplifying and sequencing the ITS-5.8S rDNA locus and performing BLAST searches as well as by performing RFLP analysis on the amplicons. RFLP analysis was performed with two restriction endonucleases, *Cfo*I and *Hae*III. The results in Figure 3.15 confirm that the strains were all *M. pulcherrima*.

In order to evaluate the presence of *MpAPr1*, PCR was performed on the gDNA of the twelve strains with the *MpAPr1* amplifying primers (MpAPr1-F/MpAPr1-R). The gene appears to be present in all the strains as seen in Figure 3.16. Extracellular acid protease activity of the twelve *M. pulcherrima* strains was assessed using a plate assay. Yeasts, grown to stationary growth phase in YPD broth, were spotted on skim milk plates at pH 3.5. Enzymatic activity was visualised by a clear halo of at least 1 mm around the edges of the yeast colony. Assays were performed in triplicate. Activity could be visualized in all the strains; however strain IWBT Y1123 had a greater activity than the rest of the strains indicated by a wider halo (Figure 3.16). VIN13, used as negative control, displayed no activity at all.

The results showed a positive correlation between the presence of the gene, confirmed by PCR, and protease activity screening on plates. However, activity was not equal amongst the strains as illustrated by plate assays. The PCR fragments of seven of the putative genes were sequenced and aligned with the sequence of IWBT Y1123 (data not shown). Only the sequence of one strain, IWBT Y1065 showed distinguishable polymorphism with 1.2% nucleotide differences compared to the other sequences. However, the deduced protein products revealed only one amino acid difference, $Val^{11} \rightarrow Ile$.

### 3.4 DISCUSSION

In this study, the putative gene and amino acid sequences of two aspartic proteases were retrieved and characterized by *in silico* analysis. The first putative gene was from *M. pulcherrima* IWBT Y1123 and the other from *C. apicola* IWBT Y1384. The putative proteins were predicted to have molecular weights of 40.8 kDa and 39.1 kDa, respectively. The molecular weight of the protein from strain IWBT Y1123 without the signal peptide (mature protease) was predicted to be 39.2 kDa. These values are in line with what has been published previously with the sizes of aspartic proteases ranging from 35 kDa to 50 kDa (Davies, 1990; Rao et al., 1998). The putative genes were named *MpAPr1* isolated from IWBT Y1123 and *CaAPr1* isolated from IWBT Y1384. The predicted pI values of the two putative proteins were 4.22 and 4.33 which are also in line with what has been reported in literature (Rao et al., 1998; Tyndall et al., 2005).

By performing homology searches, it was found that both gene sequences shared homology with genes from various species, including fish, insects and humans. The coverage scores, although low, revealed high identity with the database sequences. These conserved regions correspond to the active site-encoding regions on the putative protein sequences. It appears that the active site-encoding regions, especially that which is located on the N-terminal end are highly conserved throughout different species and indicates how the enzymes may have evolved throughout different species but have most probably retained their function. This is expected seeing that aspartic acid proteases are found in almost all living organisms: viruses, bacteria, plants, mammals etc. (Davies 1990).

No signal peptide could be detected in the CaAPr1 protein and it was predicted that the putative protein is also not a glycosylphosphatidylinositol(GPI)-anchored protein (data not shown). Although a signal peptide was detected for the DNA deduced Axp aspartic protease of *Y. lipolytica* 148, the prepro-region of the protein shared no homology with other extracellular proteins and the secretory motif of the protein was distinct from the common motif for yeast extracellular protease processing (Young et al.; 1996). McEwen and Young (1998) could not confirm whether the Axp precursor contains the signal peptide but it was confirmed that Axp translocation occurs co-translationally. Thus the signal peptide could have been cleaved co-translationally. Nonetheless the translocation process of Axp is still unknown. Because of the close homology of the CaAPr1 putative protein to the aspartic protease of *Y. lipolytica*, it may be suggested that the two proteins could follow a similar secretion and maturation pathway which is yet to be elucidated (Beckerich et al., 1998), or because of the lack of an identifiable signal peptide, the putative CaAPr1 protein may present a novel translocation process.

The putative gene *MpAPr1* was ligated into pCEL13, carrying the constitutive *PGK1* promoter, and expressed in *S. cerevisiae* YHUM272. Activity assays were performed on plates supplemented with skim milk. A zone of clearance confirmed positive activity. The activity assays confirmed that the *MpAPr1* gene encodes an extracellular aspartic protease. The zone of clearance was much wider in the IWBT Y1123 strain (native host) compared to the transformed YHUM272 strain, indicating weaker activity in the recombinant strain. This could be due to a number of factors: the differences in metabolic machinery of *S. cerevisiae* YHUM272 and *M. pulcherrima* IWBT Y1123. *S. cerevisiae* YHUM272 may not be systematically recognizing the (unfamiliar) secretion signal peptide of the protein, or the signal peptide is cleaved improperly. Expression may be lower under the *PGK1* promoter of the plasmid. *PGK1* gene expression is activated when yeast cells are grown on glucose, while *PGK1* mRNA levels are low when growth is on lactate (Chambers et al., 19898; Moore et al., 1991). The skim milk media contained lactate and had a glucose concentration of 0.8%. Due to the presence of lactate from the skim milk powder and the low glucose concentration, decreased activation of the *PGK1* promoter is possible. In 1987, Mellor et al. suggested that the absence of a downstream activating sequence (DAS) in expression vectors can explain in part the low yield of foreign proteins expressed under the control of the *PGK1* promoter when compared to the endogenous levels of 3-phosphoglycerate kinase (PGK). Further investigation is needed to elucidate the decreased expression in the recombinant strain. Figure 3.10 shows that the colonies of the recombinant *S. cerevisiae* YHUM272 carrying the *MpAPr1* gene and that of IWBT Y1123 was slightly bigger than the colonies of the strains not carrying the gene. In order to assess the role of *MpAPr1* expression on nitrogen source utilization, transformed and untransformed YHUM272 strains were spotted on skim milk media not containing the essential amino acids tryptophan, leucine and histidine. The cells not carrying the *MpAPr1* gene did not grow at all while the *MpAPr1*-transformed strain grew slightly and displayed minimal protease activity (results not shown). This indicates the important role extracellular proteolytic activity can play in the survival of yeasts under poor carbon and/or preferred nitrogen conditions.

*M. pulcherrima* IWBT Y1123 was grown in media containing different nitrogen sources (casein, BSA, grape juice proteins and ammonium sulphate) in order to evaluate induced expression of the *MpAPr1* gene. RT-PCR established that the gene is strongly expressed in the presence of casein and grape juice proteins, and only slightly in the presence of BSA. The strongest expression of the gene appeared to be in the presence of grape proteins. Quantitative real-time PCR could be performed in future in order to quantify *MpAPr1* expression under different growth conditions, particularly different nitrogen sources. The presence and activity of the MpAPr1 protease was assessed by performing SDS-PAGE analysis and zymography on cell-free concentrated supernatants. Proteolysis was observed in the SDS-PAGE analysis of the BSA induced culture and activity was observed in the grape protein-induced culture by zymography. Sequencing of the protein bands corresponding to the expected size of the mature enzyme from

casein-induced and grape protein-induced cultures further confirmed the presence of the MpAPr1 protein in the extracellular media. According to literature, protease secretion in yeasts is induced by the presence of proteins as sole nitrogen source in the extracellular medium (Dabas and Morschhäuser, 2008). It was not a surprise that the production and secretion of the acid protease is induced by grape proteins seeing that these are present in the (natural) environment from which the yeast was isolated, and may be a familiar source of nitrogen for the yeast. Casein is a commonly used inducer for proteases from yeasts and fungi (Banerjee et al., 1991; Gotoh et al., 1994), and BSA has also been used in a number of studies to induce the production and secretion of proteases (Lagace and Bisson, 1990; Togni et al., 1996). Proteins are considered alternative or secondary nitrogen sources for yeasts whereas amino acids, ammonium, glutamine and urea are preferred nitrogen sources (Banerjee et al., 1991). Therefore, the presence of the protease was expected in the cultures containing BSA, casein and grape juice proteins and not with ammonium sulphate. The genes encoding proteases are repressed when high concentrations of the preferred nitrogen sources are available (Dabas and Morschhäuser, 2008). The GATA transcription factors, which control the use of alternative nitrogen sources, have been studied extensively in *S. cerevisiae* and *C. albicans* (Marzluf et al., 1997; Morschhäuser, 2011). Investigating the presence and role of these transcription factors in *M. pulcherrima* would broaden our understanding of the expression of *MpAPr1*.

It appears that the *MpAPr1* protease gene has no introns seeing that the predicted size of the mature enzyme correspond to the size (~39 kDa) of the protein band excised from the SDS-PAGE gel. This is also an indication of the low level of *N*-glycosylation of the protein hence the ease of migration through the gel. Only one potential *N*-glycosylation site was predicted for the deduced protein sequence.

In 1994, Gotoh and co-workers reported on the purification of an acid protease from *C. pulcherrima* KSY 188-5 (teleomorph *Metschnikowia*) with a molecular weight of 36.5 kDa estimated by SDS-PAGE and gel filtration, and a pI of 4.7 determined by isoelectric focusing, which is similar to what has been found in this study for the protein of *M. pulcherrima* IWBT Y1123, 39.2 kDa and pI 4.22, respectively. The enzyme also had a wide substrate specificity hydrolysing casein, BSA, haemoglobin and collagen. Further characterization of MpAPr1 is needed to confirm whether it is identical to the enzyme described by Gotoh et al. (1994). Sequencing the gene encoding the protease from *C. pulcherrima* KSY 188-5 would also enable one to better compare the two proteases. An acid protease gene of *M. reukaufii* W6b has also been characterized (Li et al., 2009). This gene is 1527 bp long without any introns and encodes a 508 amino acid long protein with an estimated molecular weight of 53.5 kDa and a predicted pI of 4.2. The molecular weight of this protein is somewhat higher than the usual molecular weight of yeast aspartic proteases, but had the same pI of the MpAPr1 protease.

The presence of *MpAPr1* and extracellular protease activity were confirmed in 12 *M. pulcherrima* strains isolated from grape juice. As stated previously, activity was not equal amongst the strains as illustrated by plate assays. An alignment of seven of the putative protease encoding genes with that of IWBT Y1123 revealed that the sequence from strain IWBT Y1065 had 1.2% nucleotide differences compared to the other sequences. The deduced amino acid sequence of the putative gene had only one difference, $Val^{11} \rightarrow Ile$, compared with the other sequences. These two amino acids, valine and isoleucine, are both aliphatic and non-polar and a substitution between the two is unlikely to affect protein activity. No correlation was thus found between protein sequence and the degree of activity displayed on plates. Sequencing and examining the nucleotide regions upstream and downstream of the gene-encoding sequence may explain the varying degrees of activity.

Future work should include purification of the MpAPr1 enzyme and determination of its biochemical properties such as optimum pH and temperature of the enzyme activity. Another important aspect is to test the activity of the acid protease in wine, and whether it is able to hydrolyse wine proteins and reduce protein haze formation under oenological conditions, as potential inhibitors such as phenolic compounds and ethanol in wine may affect the activity of the protease. This study showed that MpAPr1 can digest some grape juice proteins and thus has potential to be tested for haze reduction abilities, its impact on aroma profile and whether the protease activity will lead to a non-negligible increase in available assimilable nitrogen content to be used by *S. cerevisiae* and lactic acid bacteria during alcoholic and malolactic fermentations. An increase in assimilable nitrogen content will certainly have an impact on the fermentation kinetics. Pollock et al. (2003) suggested heat treatment of the wine together with protease treatment could effectively reduce white wine haze. Other potential biotechnological applications of the enzyme include cheese manufacturing (Rao et al., 1998) and beer haze chill-proofing (Ormrod et al., 1991).

Our work has provided insight into how some non-*Saccharomyces* yeasts may survive in wine by extracellular proteolytic activity (Bossi et al., 2006). To our knowledge this the first report on the extracellular aspartic protease encoding genes of *M. pulcherrima* and *C. apicola*.

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**Table 3.1** List of strains used in this study.

| Microbe | Strain | Description/Genotype | Collection/Reference |
|---|---|---|---|
| *Saccharomyces cerevisiae* | VIN13 | Wine yeast | Anchor Yeast[a] |
| *Yarrowia lipolytica* | Y1698 | Dairy yeast | UOFS[b] |
| *Saccharomyces cerevisiae* | YHUM272 | Σ1278b *MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG* | Van Dyk et al. 2005 |
| *Aureobasidium pullulans* | Y1008 | Wine yeast-like fungus | IWBT[c] |
| *Candida apicola* | Y1384 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | L0642 | Wine yeast | FOEB[d] |
| *Metschnikowia pulcherrima* | Y1123 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1072 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1065 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1063 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1094 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1325 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1213 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1207 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1125 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1120 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1108 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1115 | Wine yeast | IWBT |
| *Metschnikowia pulcherrima* | Y1337 | Wine yeast | IWBT |
| *Escherichia coli* | DH5α | *[F-j80lacZΔM15Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rk_, mk1) phoA supE44 thi-1 gyrA96 relA1l]* | GIBCO-Invitrogen Life Technologies, Mowbray, South Africa |

[a]Anchor Yeast: Anchor Yeast (Cape Town, South Africa)

[b]UOFS: UFS MIRCEN Yeast culture collection (Bloemfontein, South Africa)

[c]IWBT: Institute for Wine Biotechnology (Stellenbosch, South Africa)

[d]FOEB: Faculté d'Oenologie de Bordeaux (Bordeaux, France)

**Table 3.2** List of plasmids used in this study

| Vector | Description | Reference |
|---|---|---|
| pGEM®-T Easy | *Ap$^R$ LacZ* | Promega (Whitehead Scientific, CapeTown, South Africa) |
| pJET1.2 | *Ap$^R$ rep(pMB1) eco47IR* | Fermentas (Inqaba Biotec, South Africa) |
| pCEL13 | *Ap$^R$ URA3 PGK1$_P$–PGK1$_T$* | Gundllapalli et al., 2006 |

**Table 3.3** List of primers used in this study

| Primer name | Primer sequence (5'-3')* | Source |
|---|---|---|
| ITS1 | TCCGTAGGTGAACCTGCGG | White et al., 1990 |
| ITS4 | TCCTCCGCTTATTGATATGC | White et al., 1990 |
| Degenerate primers: | | |
| AXP1 forward | GAYACNGGNTCNTCNGAY | This study |
| AXP1 reverse | NGANGTNGCNGARTCNAR | This study |
| Inverse primers: | | |
| Mpulch_ IPCR_ F1 | CAGATCTCGGCAAGGGCTCGTTGAT | This study |
| Mpulch_ IPCR_ R1 | TGCGCAAACTGGAAATTGGAAAGCA | This study |
| Mpulch_ IPCR_ F2 nested | GGTGAACGCCCCAGTTCT | This study |
| Mpulch_ IPCR_ R2 nested | AGCTGGAACTGTCTGGGCTA | This study |
| Capi_IPCR_F1 | CAGTGCCCAGAATGGAAGCAGTGTG | This study |
| Capi_IPCR_R1 | ACACCGGCAGTTTCGCCCTGAGTAG | This study |
| Capi_IPCR_F2 nested | TGGTAACAAGTCGAATGGTGTG | This study |
| Capi_IPCR_R2 nested | CCTTGAAGTTTGTCTGATTTGTG | This study |
| Primers for full gene : | | |
| MpAPr1-F | GGATCCATGCAATTCCTCACTCTTCTTTC | This study |
| MpAPr1-R | CTCGAGTTAAGCACTTATGATGTTTGACGA | This study |
| CaAPr1-F | GGA TCCATGGTACTAGCTAAGAACTATGTT CATTTA | This study |
| CaAPr1-R | CTCGAGTTAGTTGACAGATCCGGGAAT | This study |
| 5′-KPNPGK-631 | GGGGTACCCTTTATTTTGGCTTCACCC | Volschenk et al., 2004 |
| NL4 | GGTCCGTGTTTCAAGACGG | Kurtzman and Robnett, 1998 |
| NL1 | GCATATCAATAAGCGGAGGAAAAG | Kurtzman and Robnett, 1998 |

Underlined sequences indicate restriction sites. GGATCC = *BamH*I, CTCGAG = *Xho*I, GGTACC = *Kpn*I

**Table 3.4** PCR amplification programmes

| Primer pair | Initial denaturation Temp (˚C)/time (min) | Main cycling conditions | | | | Final extension Temp(˚C)/time (min) |
|---|---|---|---|---|---|---|
| | | Number of cycles | Denaturing Temp(˚C)/time (sec) | Annealing Temp(˚C)/time (sec) | Extension Temp(˚C)/time (min) | |
| ITS1/ITS4 | 95/5 | 40 | 95/1 | 58/1 | 72/1 | 72/7 |
| AXP1 forward/ reverse | 94/7 | 45 | 94/30 | 50/60 | 72/0.6 | 72/7 |
| Mpulch_ IPCR_ F1/R1 | 94/2 | 30 | 94/20 | 68/20 | 68/5 | 68/5 |
| Capi_IPCR_F1/ R1 | 94/2 | 30 | 94/20 | 60/20 | 68/5 | 68/5 |
| Mpulch_ IPCR_ F2/R2 nested | 94/2 | 30 | 94/20 | None | 68/5 | 68/5 |
| Capi_IPCR_F2/R2 nested | 94/2 | 30 | 94/20 | None | 68/5 | 68/5 |
| MpAPr1-F/R | 98/0.5 | 35 | 98/10 | 58/20 | 72/0.5 | 72/7 |
| CaAPr1-F/R | 98/0.5 | 35 | 98/10 | 58/20 | 72/0.5 | 72/7 |
| 5′-KPNPGK-631/Mpulch_ IPCR_ R2 | 96/2 | 35 | 94/30 | 58/30 | 72/0.6 | 72/10 |
| NL4/NL1 | 98/0.5 | 35 | 98/10 | 58/20 | 72/0.5 | 72/7 |

**Table 3.5** Characteristics of the putative gene and amino acid sequences of the aspartic proteases of *M. pulcherrima* IWBT Y1123, *MpAPr1* and *C. apicola* IWBT Y1384, *CaAPr1*

| Characteristic | IWBT Y1123 | IWBT Y1384 |
|---|---|---|
| **Gene length (base pairs)** | 1137 | 1101 |
| **Protein sequence length (amino acids)** | 378 | 367 |
| **Theoretical molecular weight (Dalton)** | 40885.61 | 39139.06 |
| **Theoretical pI** | 4.22 | 4.33 |
| **Signal peptide** | Position 1 – 16 | None (Non-classical secretion) |
| **Cleavage site** | Between 16 and 17 (Glycine-Methionine) | None |
| ***N*-glycosylation sites** | Position 43 | Positions 73, 188, 226 |
| **Catalytic motifs** | Positions 90–102, 257–269 | Positions 59-70, 230-241 |
| **Fully conserved catalytic regions** | 93-96 (DTGS), 260-263 (DSGT) | 62-65 (DTGS), 233-236 (DSGT) |
| **Active site flap** | Positions 122 - 133 | Positions 97 – 107 |
| **Protease family** | Eukaryotic aspartyl protease (A1 family) EC3.4.23 | Eukaryotic aspartyl protease (A1 family) EC3.4.23 |

**Table 3.6** Proteins from the NCBI database showing similarity to the deduced protein sequence of MpAPr1

| Accession number | Description | Coverage (%) | Identities (%) | Expect value | Maximum score | Gaps |
|---|---|---|---|---|---|---|
| XP_002615916.1 | *Clavispora lusitaniae* ATCC 42720, Eukaryotic aspartyl protease | 100 | 56 | 8e-127 | 377 | 17/395 (4%) |
| XP_002548135.1 | *Candida tropicalis* MYA-3404, Candidapepsin precursor | 97 | 39 | 7e-63 | 213 | 40/395 (10%) |
| 1J71_A | *Candida tropicalis,* Structure Of Extracellular Aspartic Proteinase | 79 | 41 | 7e-58 | 198 | 29/325 (9%) |
| XP_002421073.1 | *Candida dubliniensis* CD36, Putative secreted aspartyl protease, | 97 | 36 | 3e-55 | 193 | 43/399 (11%) |
| XP_711061.1 | *Candida albicans* SC5314, Secretory aspartyl proteinase SAP2p | 97 | 38 | 6e-55 | 192 | 52/405 (13%) |
| EHA18836.1 | *Aspergillus niger* ATCC 1015, Aspartic protease | 97 | 34 | 2e-46 | 170 | 54/407 (13%) |
| XP_501603.1 | *Yarrowia lipolytica* CLIB122, Eukaryotic aspartyl protease | 85 | 33 | 1e-37 | 146 | 42/354 (12%) |

**Table 3.7** Proteins from the NCBI database showing similarity to the deduced protein sequence of CaAPr1

| Accession number | Description | Coverage (%) | Identities (%) | Expect value | Maximum score | Gaps |
|---|---|---|---|---|---|---|
| XP_500342.1 | *Yarrowia lipolytica* CLIB122, Eukaryotic aspartyl protease | 92 | 37 | 2e-54 | 190 | 34/364 (9%) |
| XP_499671.1 | *Yarrowia lipolytica* CLIB122, Eukaryotic aspartyl protease | 94 | 37 | 5e-53 | 188 | 36/372 (10%) |
| XP_002548135.1 | *Candida tropicalis* MYA-3404, Candidapepsin precursor | 89 | 40 | 5e-53 | 187 | 35/351 (10%) |
| XP_001398592.1 | *Aspergillus niger* CBS 513.88, Aspartic-type endopeptidase opsB | 93 | 36 | 5e-51 | 183 | 44/379 (12%) |
| XP_720071.1 | *Candida albicans* SC5314, Secretory aspartyl proteinase SAP8p | 87 | 36 | 3e-43 | 161 | 47/348 (14%) |
| XP_456485.2 | *Debaryomyces hansenii* CBS767, Eukaryotic aspartyl protease | 83 | 34 | 2e-38 | 149 | 74/369 (20%) |
| EEU06988.1 | *Saccharomyces cerevisiae* JAY291, Yps1p Eukaryotic aspartyl protease | 78 | 37 | 2e-37 | 149 | 41/317 (13%) |

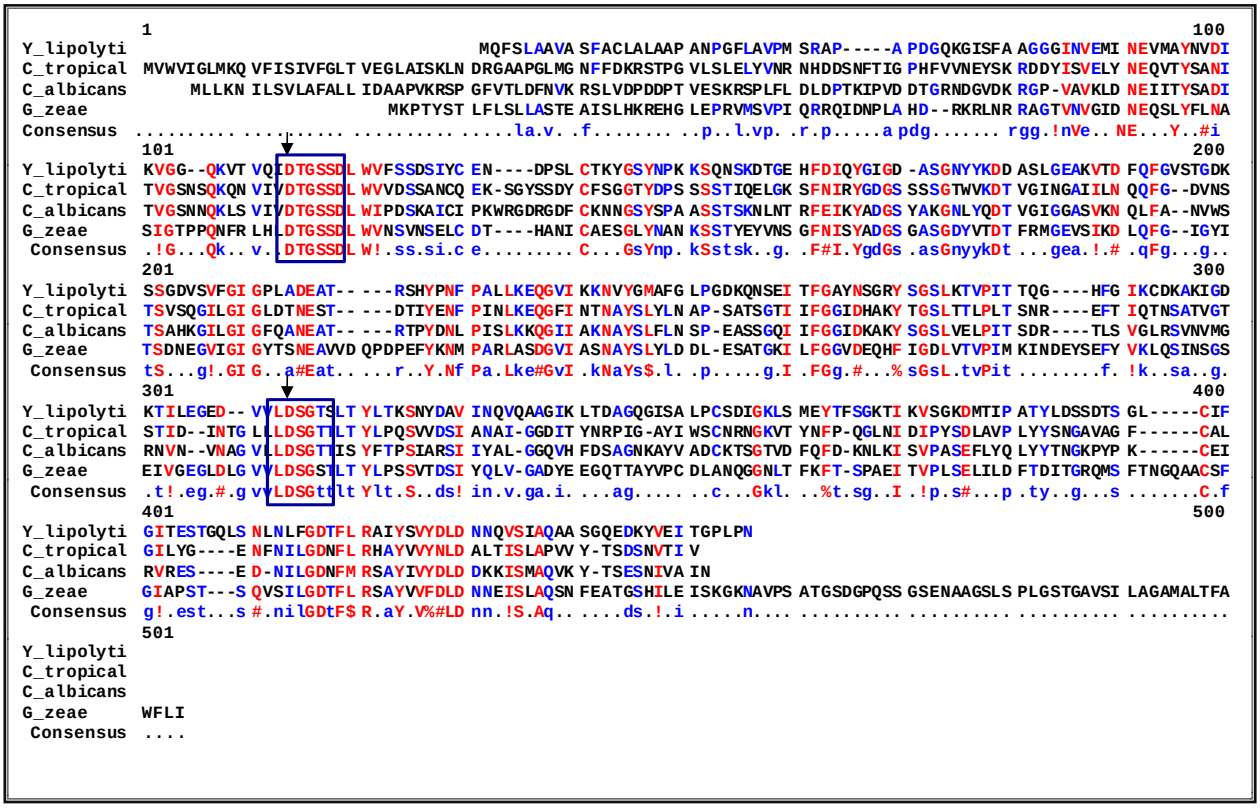


**Figure 3.1** Alignment of the protein sequences of the extracellular acid protease of *Y. lipolytica* CLIB122 and those of three other yeasts. Y_lipolyti, *Yarrowia lipolytica*; C_tropical, *Candida tropicalis*; C_albicans, *Candida albicans*; G_zeae, *Gibberella zeae*. *Boxed* sequences indicate the active site regions used to design degenerate primers. The *arrows* indicate the aspartic acid residues of the active sites. Residues in red represent high consensus, residues in blue low consensus and residues in black, neutral. All the characters in upper case in the consensus sequence are identical with the consensus sequence, and those in lower case are either the most likely residue or a homologous symbol. The small symbols represent different homologies according to the Blosum62-12-2 symbol comparison table. The different symbols that are homologous are: (!) for I and V; ($) for L and M; (%) for F and Y; (#) for B, D, E, N, Q and Z. The point (.) represents the residue identical to the first sequence at the same position.

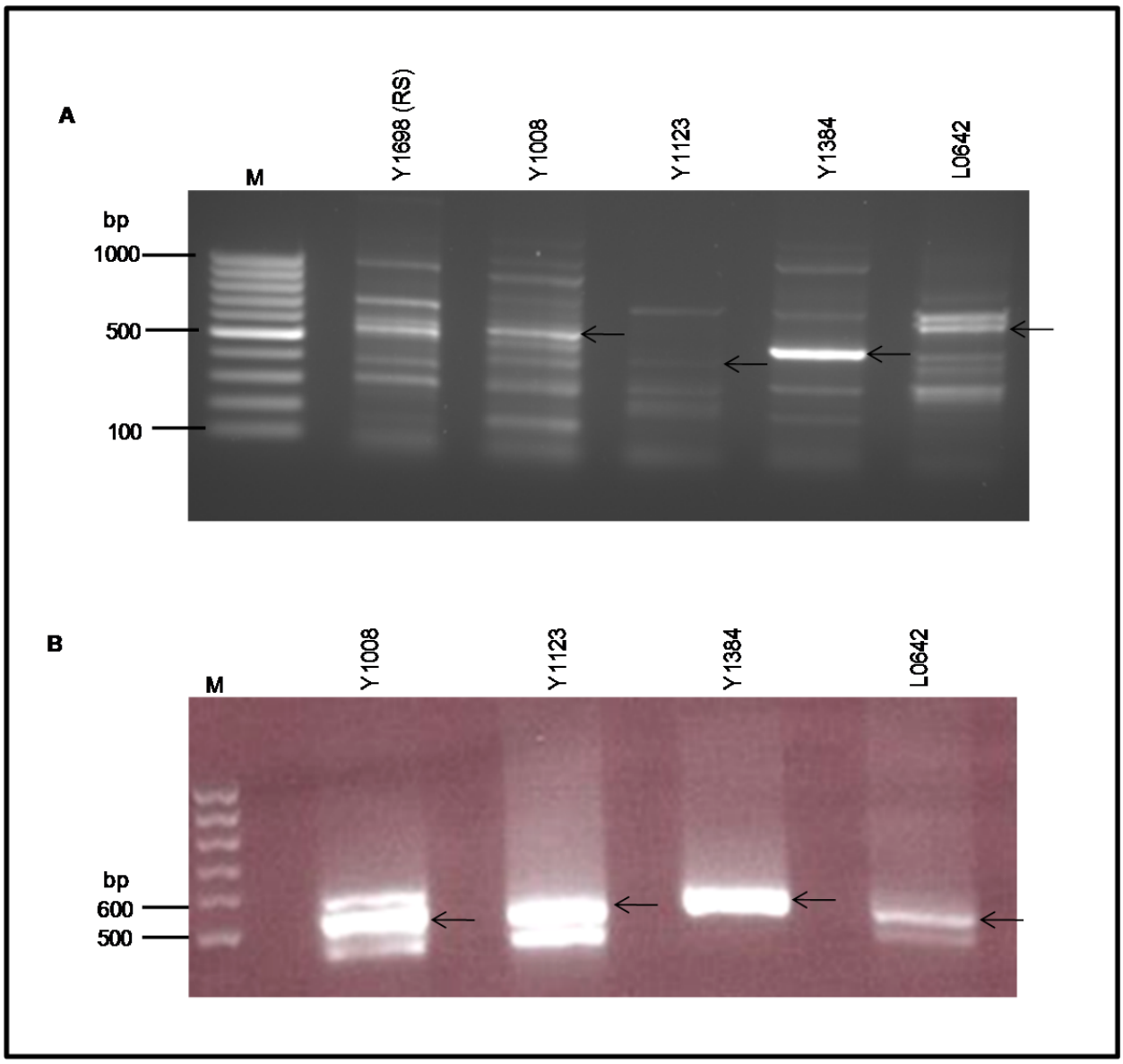


**Figure 3.2** PCR amplification with degenerate primers. (**A**) First round of amplification. (**B**) Amplification of the excised bands at approximately 570 bp. *Y. lipolytica* UOFS-Y1698 used as reference strain (RS). Arrows indicate excised and sequenced bands. M, molecular weight marker, GeneRuler$^{TM}$ 100bp DNA ladder (Fermentas).

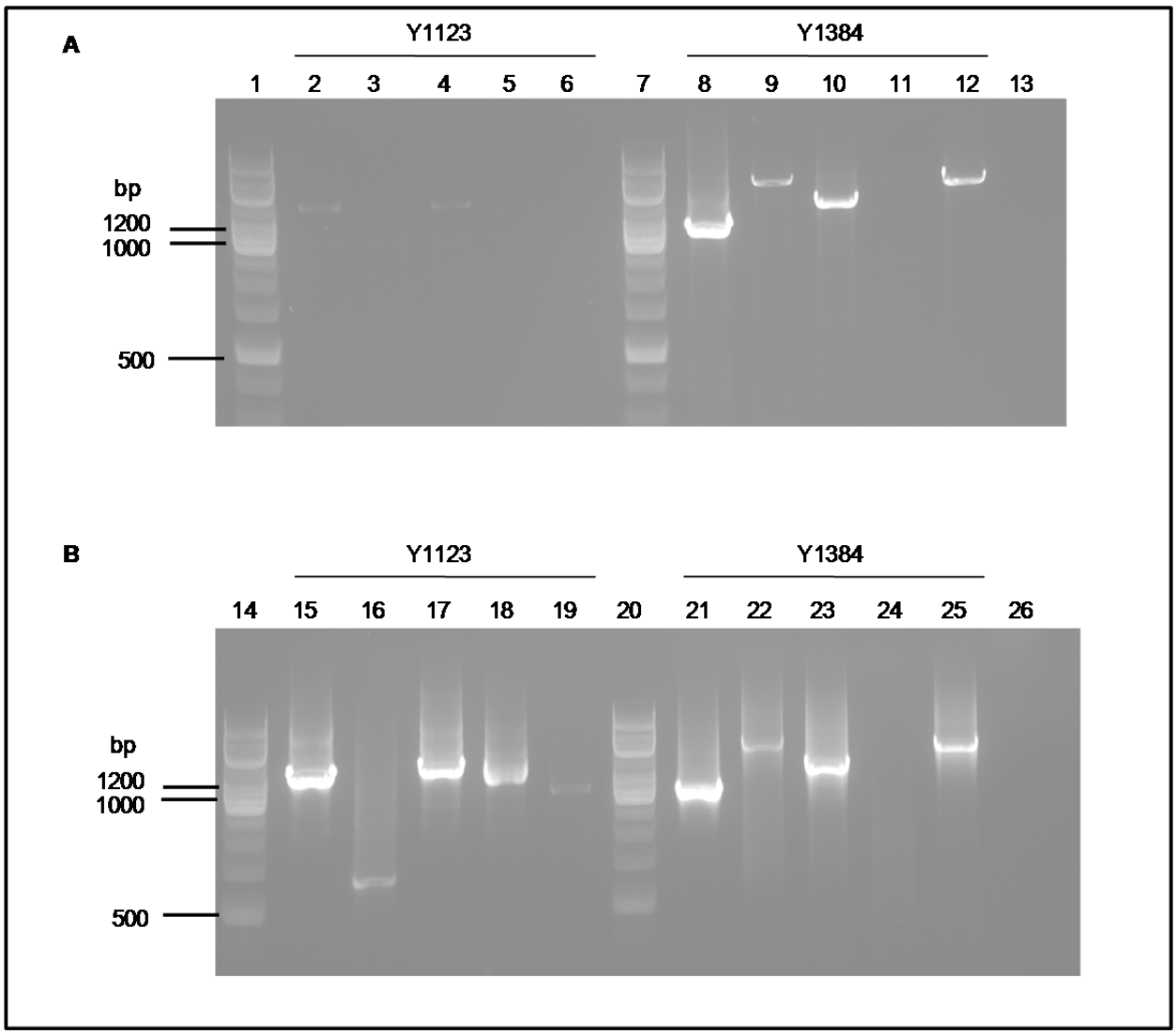


**Figure 3.3** Inverse-PCR amplification. PCR performed on gDNA which was digested with different restriction enzymes. (**A**) Amplification with external primers. (**B**) Amplification with nested primers. Lanes 2, 8, 15, 21 was performed with gDNA digested with *Eco*RI; Lanes 3, 9, 16, 22 gDNA digested with *Dra*I; Lanes 4, 10, 17, 23 gDNA digested with *Eco*RV; Lanes 5, 11, 18, 24 gDNA digested with *Hpa* I; Lanes 6, 12, 19, 25 gDNA digested with *Xba*I. Lane 26 is the PCR negative control. Lane 1,7,14, 20 are molecular weight markers, GeneRuler™ 100bp DNA ladder Plus (Fermentas).

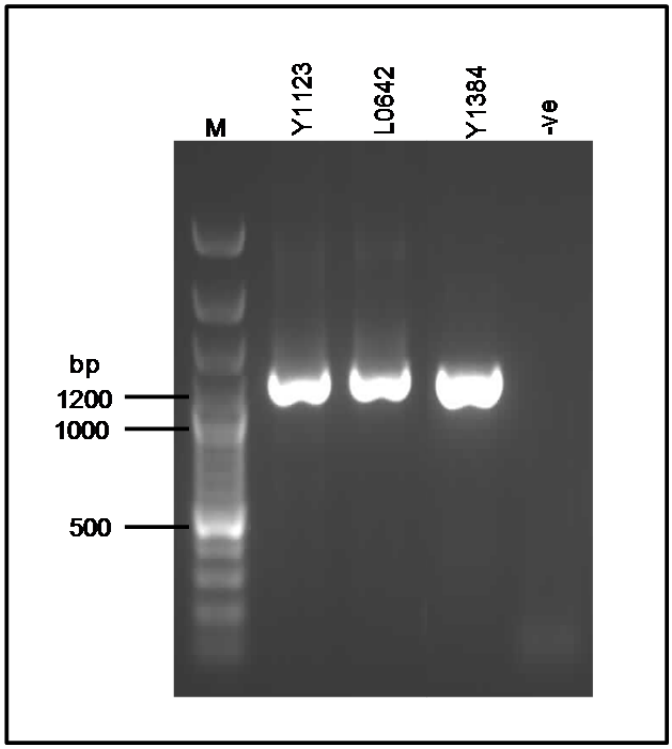


**Figure 3.4** PCR amplifications indicating the sizes of the full genes. (-ve) PCR negative control. M is the molecular weight marker, GeneRuler$^{TM}$ 100bp DNA ladder Plus (Fermentas).

```
   1 ATGCAATTCCTCACTCTTCTTTCTCTTGCAGTCGCTCTCGTCAACGGCATGGCCATCCCTGGGCTTGACACA 72
   1 M  Q  F  L  T  L  L  S  L  A  V  A  L  V  N  G‡ M  A  I  P  G  L  D  T   24

  73 CGCGACGAGGCGGCGCCTTTGCAGCTTGACTTCACCGTGCTGAAAACAGTTGGCAACACGACGGCAAAGGAG 144
  25 R  D  E  A  A  P  L  Q  L  D  F  T  V  L  K  T  V  G  N  T  T  A  K  E   48

 145 TTCTGGGCGAAATACGGATCAAAAAATAAGAAGCGTGACGCGTACCCGGAAGTGATCACGGACTATCGCGAC 216
  49 F  W  A  K  Y  G  S  K  N  K  K  R  D  A  Y  P  E  V  I  T  D  Y  R  D   72

 217 TTGAGCTACCAGATCGATGTGTACTTGGGCGCAGATAAACAGAAGAATACCGTTTCCTTGGATACGGGCTCG 288
  73 L  S  Y  Q  I  D  V  Y  L  G  A  D  K  Q  K  N  T  V  S  L  D  T  G  S   96

 289 TCGGATCTTTGGGTGCCCAGCAGCGGCTATAGCCCAGACAGTTCCAGCTCTGCACAGGACACCGGTGAAGCA 360
  97 S  D  L  W  V  P  S  S  G  Y  S  P  D  S  S  S  S  A  Q  D  T  G  E  A  120

 361 TTCAAAATTGGATATTTGGATGGAAGCGGTGCACTGGGTGAATATTACAAGGACAAGTTCCAGTTCAGCACA 432
 121 F  K  I  G  Y  L  D  G  S  G  A  L  G  E  Y  Y  K  D  K  F  Q  F  S  T  144

 433 GCCAAGCCAGTGCTTTCCAATTTCCAGTTTGCGCAGACTAGCGACGAGGCGGGCATGGGGATTCTAGGCATT 504
 145 A  K  P  V  L  S  N  F  Q  F  A  Q  T  S  D  E  A  G  M  G  I  L  G  I  168

 505 GCCGACCGGAACCAGGAAGCGTCCGATTCGGTATATGATAATCTCCCTTGGGCATTGCAAAAAGCCGGAATC 576
 169 A  D  R  N  Q  E  A  S  D  S  V  Y  D  N  L  P  W  A  L  Q  K  A  G  I  192

 577 ACACCCAAGGCGTCATACTCGTTATTCTTGGGCCCAGATCTCGGCAAGGGGTCGTTGATTTTCGGCGGCATT 648
 193 T  P  K  A  S  Y  S  L  F  L  G  P  D  L  G  K  G  S  L  I  F  G  G  I  216

 649 GACACAGACAAGTATACTGGAGAGTTGCAGCTGTACCCGATTGACCTGTCCAGCGGTGGCTTGGCCGTCGAT 720
 217 D  T  D  K  Y  T  G  E  L  Q  L  Y  P  I  D  L  S  S  G  G  L  A  V  D  240

 721 GTGCAGTCAGTGAATTTCAACGGGAAAATCATCTCGGTGAACGCCCCAGTTCTTTTGGACTCTGGAACCTCT 792
 241 V  Q  S  V  N  F  N  G  K  I  I  S  V  N  A  P  V  L  L  D  S  G  T  S   264

 793 TTGGGGCTTCTCAGCCTGGACCTTATTGAGGAGTTGGACACGATATTCGACTCTCAAACTGTGAAACAGGGT 864
 265 L  G  L  L  S  L  D  L  I  E  E  L  D  T  I  F  D  S  Q  T  V  K  Q  G   288

 865 GGGATCGAGTACAAAATTGTCAGCTGTGACCAGCCTTCAGACAAGAGCCTCGACTTTGATTTTGGCGACAAC 936
 289 G  I  E  Y  K  I  V  S  C  D  Q  P  S  D  K  S  L  D  F  D  F  G  D  N  312

 937 ACGATCTCAATTCCGTTTTCTGAGGCCATTATCAAGCAAGACGACGGCACGTGTTTGTTAGGGTTCGGATAC 1008
 313 T  I  S  I  P  F  S  E  A  I  I  K  Q  D  D  G  T  C  L  L  G  F  G  Y  336

1009 TATAATGACATTCAGATCTTCGGCGACGTGTTTTTGAGACAGGCATACGTGTACTACGATCTCACCGATAAG 1080
 337 Y  N  D  I  Q  I  F  G  D  V  F  L  R  Q  A  Y  V  Y  Y  D  L  T  D  K  360

1081 ACGATTTCGCTTGCACAGGCGTCTTATTCTAGCTCGTCAAACATCATAAGTGCTTAA 1137
 361 T  I  S  L  A  Q  A  S  Y  S  S  S  S  N  I  I  S  A  *   378
```

**Figure 3.5** The DNA and amino acid sequences of *M. pulcherrima* IWBT Y1123 putative secreted aspartic protease. The putative secretion signal peptide is underlined. The cleavage site is indicated by (‡). The active site amino acid sequences and active site flap amino acid sequence are shaded in yellow and turquoise, respectively. The putative *N*-glycosylation site is boxed.

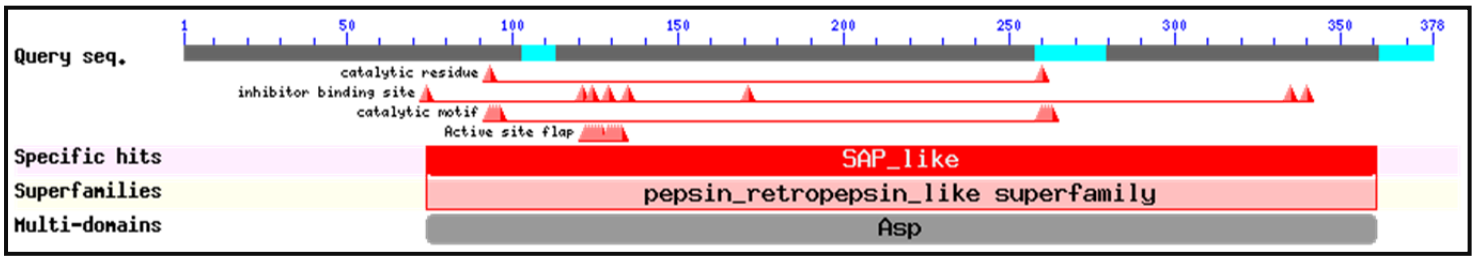


**Figure 3.6** The conserved domains within the putative aspartic protease of *M. pulcherrima* IWBT Y1123 analysed in this study, indicating that the protein is a secreted aspartic protease-like (SAP-like) enzyme.

```
   1 ATGGTACTAGCTAAGAACTATGTTCATTTAGGCATTAAGCGTGCCTTACAAGGTGATCGTACTACCGCTAAC  72
   1 M  V  L  A  K  N  Y  V  H  L  G  I  K  R  A  L  Q  G  D  R  T  T  A  N   24

  73 CGTGCACGTTCTAAGCGTGCATCCGTACCTGATCGATTGCAGAACAACATTTATCAGTACATTGCTACGTTA 144
  25 R  A  R  S  K  R  A  S  V  P  D  R  L  Q  N  N  I  Y  Q  Y  I  A  T  L   48

 145 TCAATTGGAACACCTGGCCAAGCTACTGATGCTGCACTTGATACTGGCTCCTCAGATCTGTGGGTATTCACA 216
  49 S  I  G  T  P  G  Q  A  T  D  A  A  L  D  T  G  S  S  D  L  W  V  F  T   72

 217 AATCAGACAAACTTCAAGGACAGTTTCAACCCTCAGAGTAGTTCTTCGTACAATCTGTTGAACAACGATTTT 288
  73 N  Q  T  N  F  K  D  S  F  N  P  Q  S  S  S  S  Y  N  L  L  N  N  D  F   96

 289 TCAATTCAGTATGTGTCCGGGTCAGCCAGTGGAAGCTGGGTGACAGACACGGTAGATTATGGAGATGACAAG 360
  97 S  I  Q  Y  V  S  G  S  A  S  G  S  W  V  T  D  T  V  D  Y  G  D  D  K  120

 361 GTCTCGTCGTTTCAGTTCGCAACTGTCAGCTCTCCAACTACTCAGGGCGAAACTGCCGGTGTGTTCGGTATC 432
 121 V  S  S  F  Q  F  A  T  V  S  S  P  T  T  Q  G  E  T  A  G  V  F  G  I  144

 433 GGTCAAATTACCCAAGAATCATCTGCCGAGTACGGATCTACCTACCCGAATTTCCCAGTATCTCTTAAGAAT 504
 145 G  Q  I  T  Q  E  S  S  A  E  Y  G  S  T  Y  P  N  F  P  V  S  L  K  N  168

 505 GAGTCGAAAATCCAATCTGTTGCTTATTCACTTTATTTAGACTCAATCAGTGCCCAGAATGGAAGCAGTGTG 576
 169 E  S  K  I  Q  S  V  A  Y  S  L  Y  L  D  S  I  S  A  Q  N  G  S  S  V  192

 577 ACATTTGGTGCAGTGGATACCGCGAAATATAAGGGTGAGCTTTACAGTGTCCCTTTCACCTCCGATGTGAGT 648
 193 T  F  G  A  V  D  T  A  K  Y  K  G  E  L  Y  S  V  P  F  T  S  D  V  S  216

 649 TTCAATGTTGACTTTGAAGTACTTGGTAACAAGTCGAATGGTGTGCTTGACTCTGGTACTTCATTGACCTAT 720
 217 F  N  V  D  F  E  V  L  G  N  K  S  N  G  V  L  D  S  G  T  S  L  T  Y  240

 721 TTGGAACAATCCGTTGTGGATCAAATCGCCCAGCAGTACGGAGCAACTTTCGATTCAGAGCAGCAAACTTAC 792
 241 L  E  Q  S  V  V  D  Q  I  A  Q  Q  Y  G  A  T  F  D  S  E  Q  Q  T  Y  264

 793 CTCATTCAGAGCAAGAGTGATTTAGCTAGCACTGATCCTCTGGTTTATACCATTGGAGGTGCAAAGATAGAA 864
 265 L  I  Q  S  K  S  D  L  A  S  T  D  P  L  V  Y  T  I  G  G  A  K  I  E  288

 865 GTACCTGTCTCGGAACTGTTCATCGAGGACTCTTCTGATGGTACACTGGCACTCTCTATCCTCCCTAGTTCT 936
 289 V  P  V  S  E  L  F  I  E  D  S  S  D  G  T  L  A  L  S  I  L  P  S  S  312

 937 ATGGCTCAAGATGTCATTTTACTTGGCGATAGCTTCTTGCGCTCAGCTTACGTTGTCTACAACTTACAAGGC 1008
 313 M  A  Q  D  V  I  L  L  G  D  S  F  L  R  S  A  Y  V  V  Y  N  L  Q  G  336

1009 AAAGTTGCTGGTATTGCACAAGCCAATTGGTCACCAGGCTCTCCCAACTTTGTCCCTATCACCGGAGATACT 1080
 337 K  V  A  G  I  A  Q  A  N  W  S  P  G  S  P  N  F  V  P  I  T  G  D  T  360

1081 ATTCCCGGATCTGTCAACTAA 1101
 361 I  P  G  S  V  N  *   366
```

**Figure 3.7** The DNA and amino acid sequence of *C. apicola* IWBT Y1384 secreted aspartic protease. The putative secretion signal peptide is underlined. The cleavage site is indicated by (‡). The active site amino acid sequences and active site flap amino acid sequence are shaded in yellow and turquoise, respectively. The putative *N*-glycosylation sites are boxed.

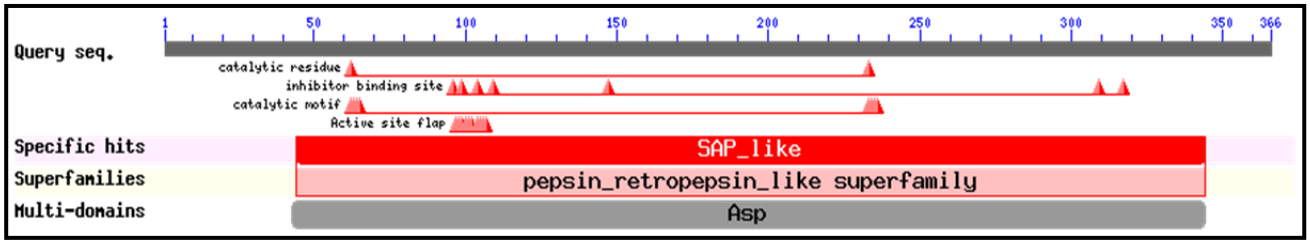


**Figure 3.8** The conserved domains within the putative aspartic protease of *C. apicola* IWBT Y1384 analysed in this study, indicating that the protein is a secreted aspartic protease-like (SAP-like) enzyme.

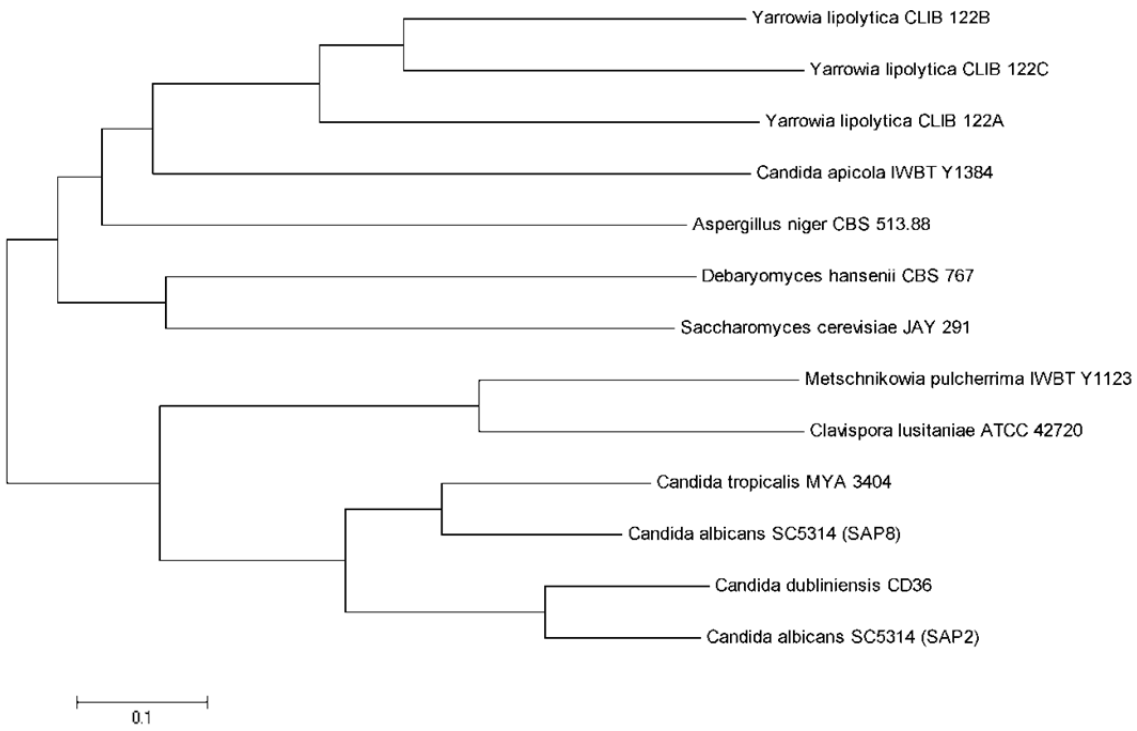


**Figure 3.9** Phylogenetic tree of the deduced protein sequences of *MpAPr1* of *M. pulcherrima* IWBT Y1123 and *CaAPr1* of *C. apicola* IWBT Y1384 with aspartic proteases from other yeasts found on the NCBI website. The phylogenetic tree was constructed by using the neighbour joining method **(**PHYLIP 3.56). The scale bar represents the number of base substitutions per site.

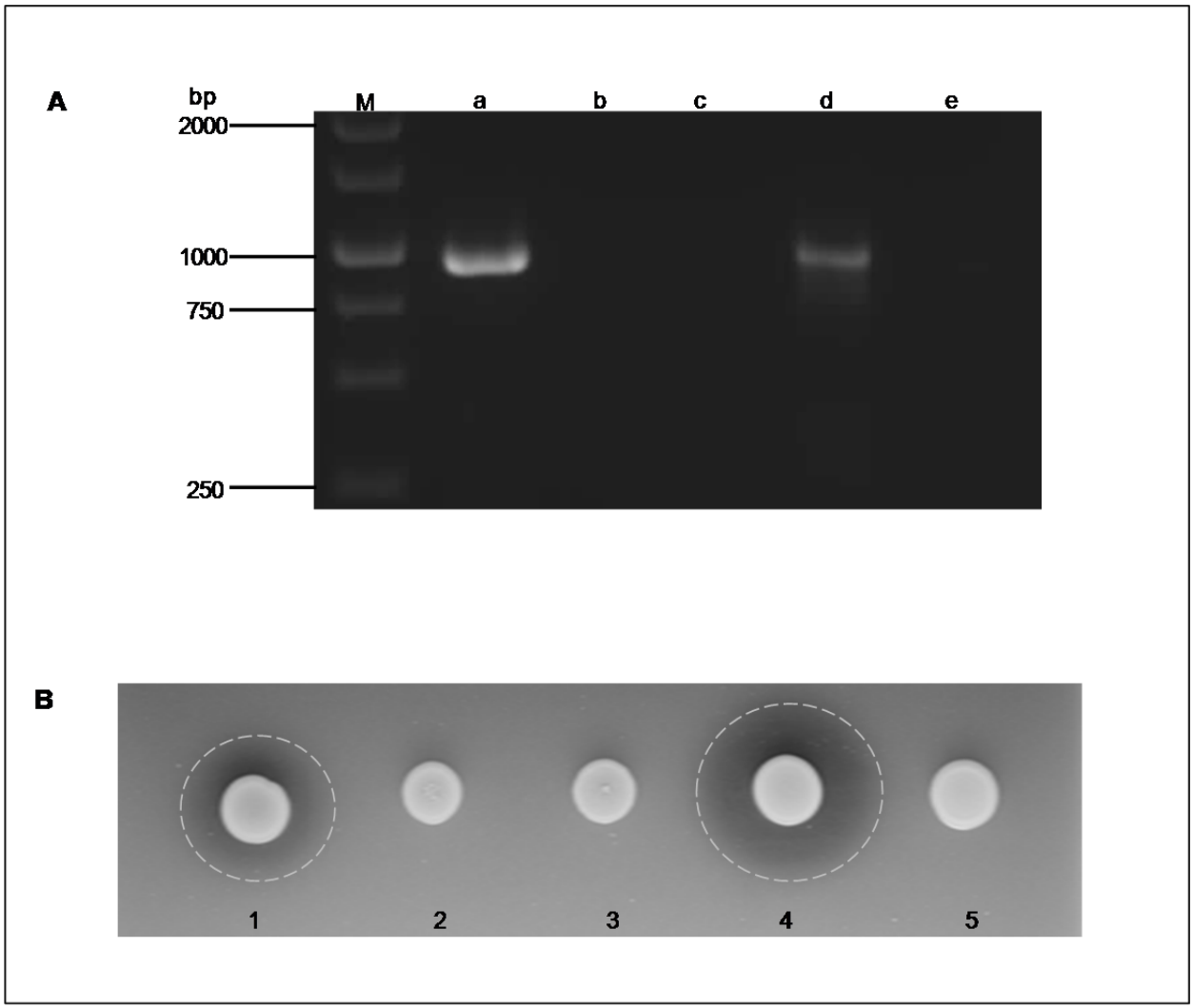


**Figure 3.10** Confirmation of heterologous expression of *MpAPr1* in *S. cerevisiae* YHUM272. (**A**) PCR amplification with primers 5′-KPNPGK-631 and Mpulch_ IPCR_ R2. (a) colony PCR of YHUM272 transformed with *MpAPr1*, (b) amplification of pCEL13 plasmid without the insert, (c) colony PCR of untransformed YHUM272, (d) amplification of pCEL13 ligated with *MpAPr1* (PCR positive control), (e) PCR negative control. M is the molecular weight marker, GeneRuler$^{TM}$ 100bp DNA ladder Plus (Fermentas). (**B**) Plate assay of extracellular protease activity. (1) recombinant YHUM272 transformed with *MpAPr1*, (2) YHUM272 transformed with empty vector, (3) untransformed YHUM272, (4) *M. pulcherrima* IWBT Y1123 (positive control), (5) *S. cerevisiae* VIN13 (negative control).

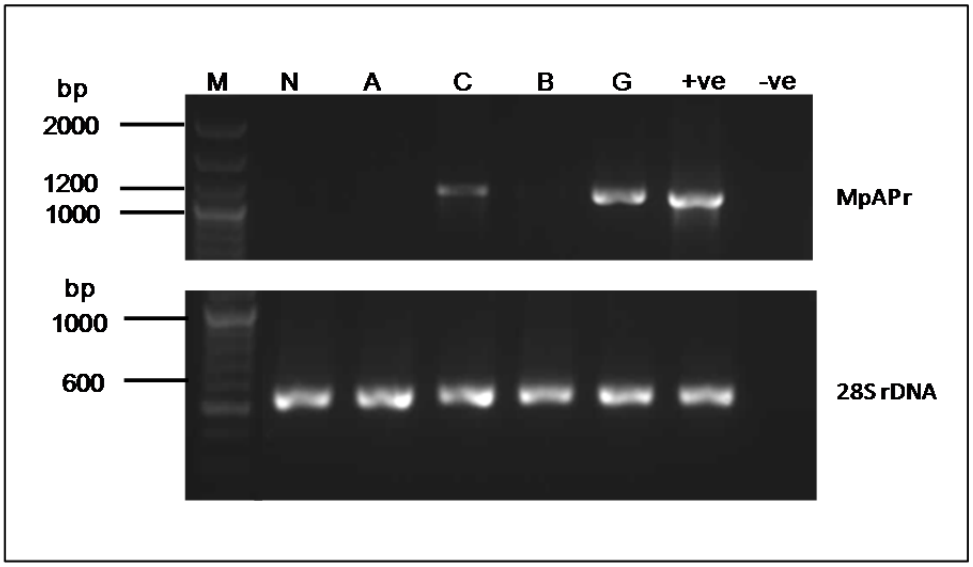


**Figure 3.11** RT-PCR analysis of the induction and expression of *MpAPr* of *M. pulcherrima* IWBT Y1123 grown in media with different nitrogen sources. (N) Culture with no nitrogen addition (blank), (A) culture with ammonium sulfate addition, (C) culture with casein addition, (B) culture with BSA addition, (G) culture with grape juice proteins addition. gDNA amplification RT-PCR positive control (+ve). RT-PCR negative control (-ve). The 28S rDNA gene was used as housekeeping gene. M is the molecular weight marker, GeneRuler$^{TM}$ 100bp DNA ladder Plus (Fermentas).

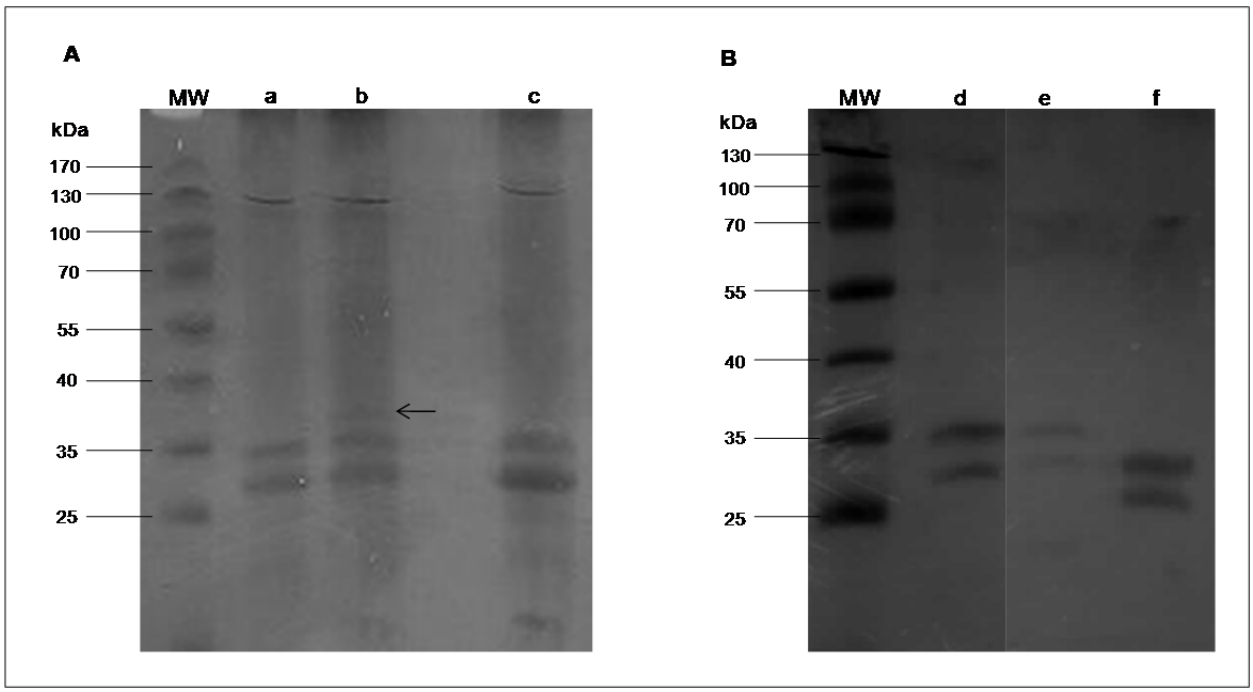


**Figure 3.12** Assessment of extracellular proteins of *M. pulcherrima* IWBT Y1123 grown in the presence of casein (**A**) SDS-PAGE analysis. Lanes: (a) casein without culture supernatant, (b) culture supernatant with casein, (c) casein after 3 day incubation without culture supernatant. (**B**) Zymograms. (d) Casein without culture supernatant, (e) Culture supernatant with casein, (f) casein after 3 day incubation without culture supernatant. MW is the molecular weight standard proteins, PageRuler$^{TM}$ Prestained Ladder (Bio-Rad). The *arrow* points to the band excised and sequenced. (Estimated according to theoretical MW is 39.2 kDa).

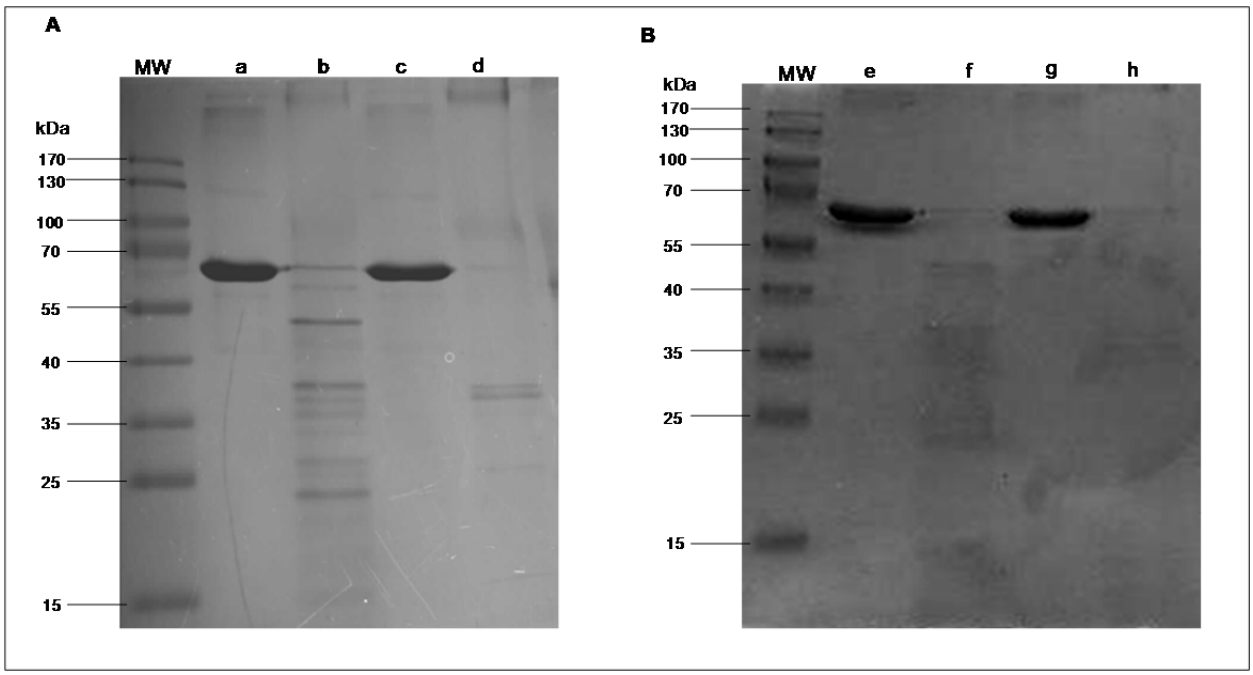


**Figure 3.13** Analysis of extracellular proteins of *M. pulcherrima* IWBT Y1123 cultured in the presence of BSA. (**A**) SDS-PAGE analysis. Lanes: (a) BSA without culture supernatant, (b) culture supernatant with BSA, (c) BSA after 3 day incubation without culture supernatant, (d) no nitrogen blank sample. (**B**) Zymogram. (e) BSA without culture supernatant, (f) culture supernatant with BSA, (g) BSA after 3 days incubation without culture supernatant, (h) no nitrogen (blank) sample. MW is the molecular weight standard proteins, PageRuler$^{TM}$ Prestained Ladder (Bio-Rad).

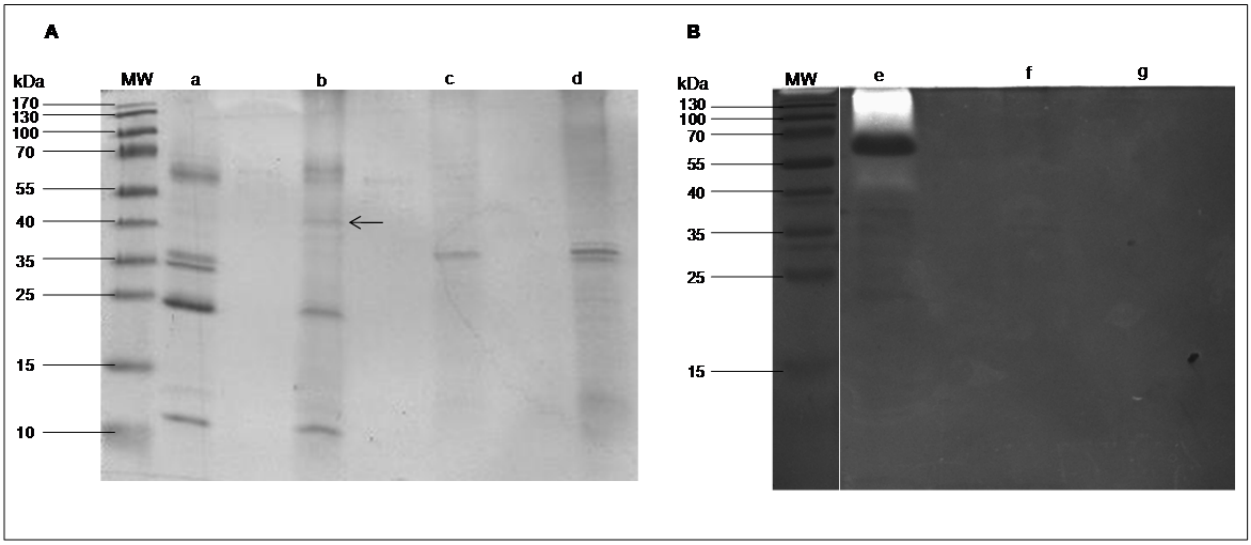


**Figure 3.14** Analysis of the extracellular proteins of *M. pulcherrima* IWBT Y1123 cultured on grape juice proteins and ammonium sulphate. (**A**) SDS-PAGE analysis. Lanes: (a) grape juice proteins without culture supernatant, (b) culture supernatant on grape juice proteins-induced, (c) culture supernatant with ammonium sulphate-induced, (d) no nitrogen (blank) sample. (**B**) Zymogram. (e) grape juice proteins culture supernatant, (f) culture supernatant with ammonium sulphate-induced, (g) no nitrogen (blank) sample. MW is the molecular weight standard proteins, PageRuler$^{TM}$ Prestained Ladder (Bio-Rad). The *arrow* points to the band excised and sequenced. (Estimated according to theoretical MW is 39.2 kDa).

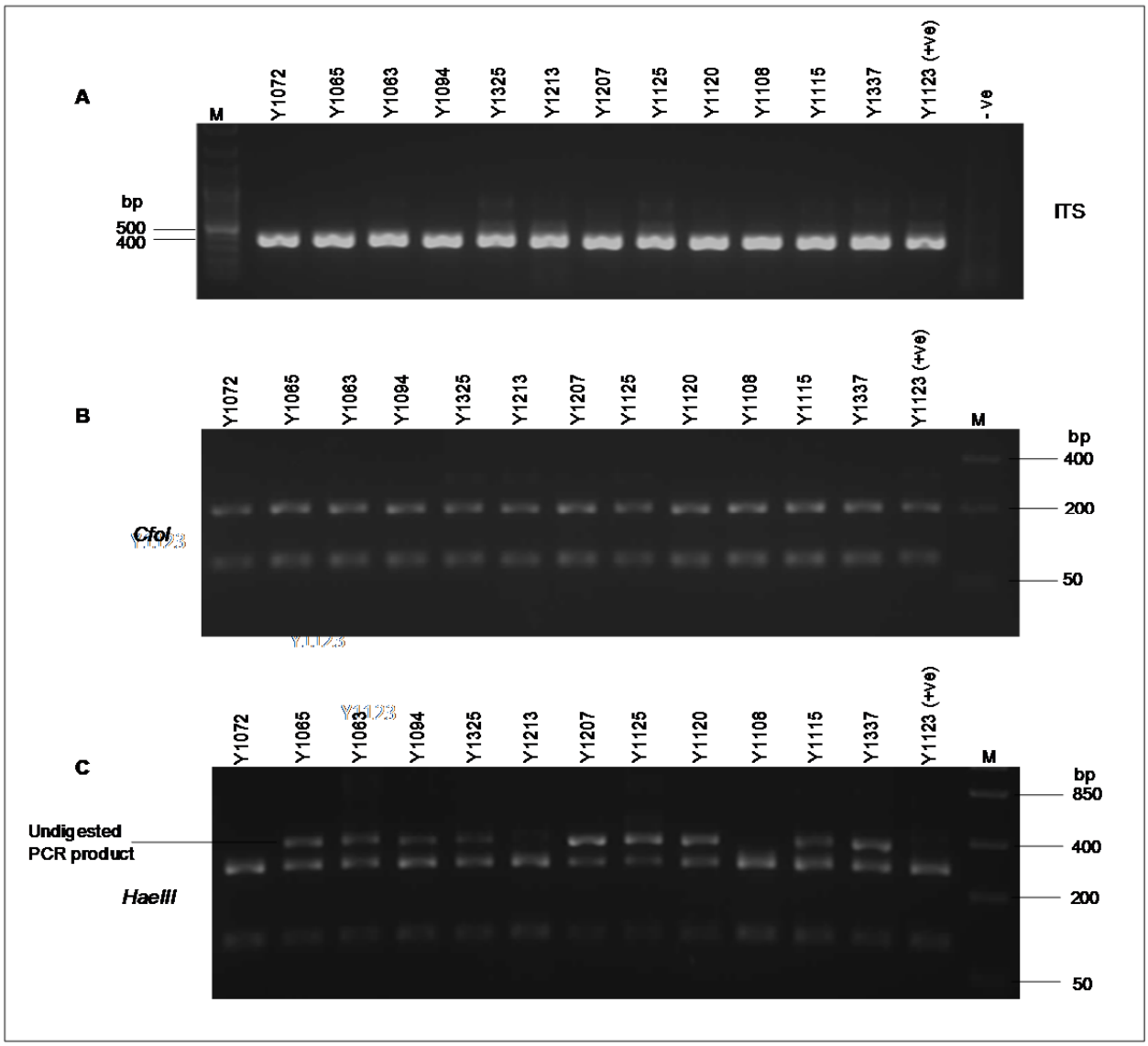


**Figure 3.15** Identification of 12 *M. pulcherrima* strains by ITS and ITS-RFLP. (**A**) PCR amplification of the ITS regions of *M. pulcherrima* strains. M, molecular weight marker, GeneRuler$^{TM}$ 100bp DNA ladder (Fermentas). (**B**) Digestion of the ITS amplifications with restriction endonuclease *Cfo*I. (**C**) Digestion of the ITS amplifications with restriction endonuclease *Hae*III. (+ve) PCR positive control in this case strain *M. pulcherrima* IWBT Y1123, (-ve) PCR negative control. M is the molecular weight marker, FastRuler$^{TM}$ Low Range DNA Ladder (Fermentas).

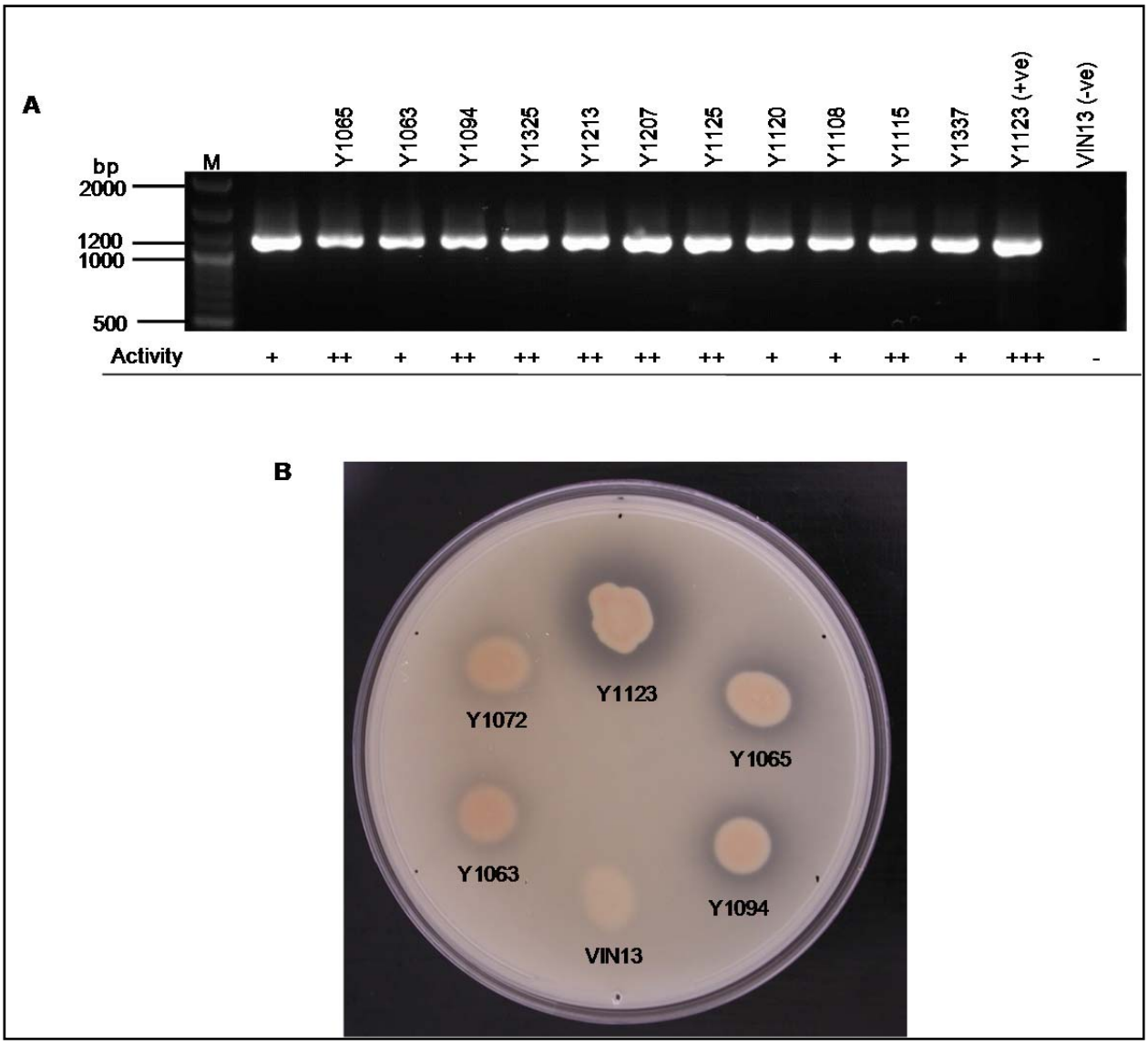


**Figure 3.16** Protease activity screening of *M. pulcherrima* strains. (**A**) PCR amplifications with primers MpAPr1-F/MpAPr1-R showing the presence of the *MpAPr1* protease-encoding gene. M is the molecular weight marker, GeneRuler™ 100bp DNA ladder Plus (Fermentas). (**B**) An example of a plate assay showing the different degrees of activity with a positive result indicated by a clear halo around the colony; (+) low activity, diameter of the halo from the edge of the colony 2mm, (++) medium activity, diameter of the halo from the edge of the colony 3 mm, (+++) high activity, diameter of the halo from the edge of the colony 5 mm, (-) no activity. Strain *M. pulcherrima* IWBT Y1123 was used as positive control (displaying high activity) and *S. cerevisiae* VIN13 as negative control (displaying no activity). Strains IWBT Y1072 and IWBT Y1063 have low activity while strains IWBT Y1065 and IWBT Y1094 show medium activity.

# Chapter 4

# General discussion and conclusions

# 4. General discussion and conclusions

## 4.1 Results and general discussion

The enzymes of the aspartic endoprotease family are defined by the presence of two aspartic acid residues in their catalytic sites that are critical for the functioning of the enzymes. These enzymes function under acidic conditions. Literature reports that some non-*Saccharomyces* yeasts of oenological origin have extracellular proteolytic activity under acidic conditions, e.g. *Candida stellata*, *Kloeckera apiculata* and *Candida pulcherrima* (Charoenchai et al., 1997). These poorly characterised enzymes could potentially fulfil a number of roles in wine production such as the reduction of protein haze in white wines (Lagace and Bisson, 1990; Dizy and Bisson, 2000). The amino acids and peptides liberated from the action of the proteases could be used as sources of nitrogen by the fermentative microorganisms. Some amino acids can be metabolised by yeasts to form aromatic compounds such as higher alcohols (Swiegers and Pretorius, 2005), thus the proteolytic activity could have an indirect impact on the aroma profile of the wine. The main aim of the study was to isolate and characterize extracellular aspartic proteases at a genetic level from non-*Saccharomyces* yeasts that are of oenological origin.

The genes encoding extracellular aspartic proteases from two non-*Saccharomyces* yeasts were isolated and characterized by *in silico* analysis. The yeasts were *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. These yeasts were isolated from grape must and showed strong proteolytic activity during an enzymatic screening of 308 other yeasts.

The genes were retrieved by following a PCR approach using degenerate primers and inverse-PCR. The gene from *M. pulcherrima* IWBT Y1123 was named *MpAPr1*. The gene is 1137 bp long. The mature form of the protein encoded by the gene consists of 362 amino acids with a predicted $M_r$ of 39.2 kDa and one possible *N*-glycosylation site. The gene also encodes a putative 16 amino acid signal peptide. The putative gene from *C. apicola* IWBT Y1384 was named *CaAPr1*, is 1101 bp long and encodes a putative protein with $M_r$ 39.1 kDa. This enzyme had no predictable signal peptide and is thought to follow a non-classical secretion pathway and had 3 possible *N*-glycosylation sites. The predicted pI values of the putative proteins were 4.22 for MpAPr1 and 4.33 for CaAPr1, respectively. These characteristics correspond to previously published data on the extracellular proteases of yeasts. The *AXP* gene of *Yarrowia lipolytica* 148 encodes a mature acid protease of 353 amino acids with a predicted $M_r$ of 39.2 kDa. The gene also encodes a putative pre-peptide that is 17 amino acids long (Young et al., 1996). *Cryptococcus* sp. S-2 possesses a *CAP1* gene with an open reading frame of 1254 bp encoding a protein of 417 amino acids long. The mature protein had an estimated $M_r$ of 34 kDa on SDS-PAGE (Rao et al., 2011).

From homology searches of the putative gene sequences, it was confirmed that the sequences encoding the conserved active sites are indeed highly conserved throughout different species (Davies, 1990; Dunn, 2002). Both putative protein sequences, MpAPr1 and CaAPr1, showed homology to only fungal aspartic proteases with average identity scores of less than 40%, indicating the novelty of the putative proteins. The CaAPr1 protein showed close homology to the aspartic proteases of *Y. lipolytica*, which appears to follow a translation pathway other than that described for *Candida* spp. (McEwen and Young, 1998; Naglik et al., 2004). CaAPr1 and the acid proteases from *Y. lipolytica* may have similar processing pathways.

The putative *MpAPr1* gene was cloned and expressed in *Saccharomyces cerevisiae* YHUM272. Activity plate assays confirmed that *MpAPr1* indeed codes for an extracellular aspartic protease. The recombinant strain demonstrated weaker activity than the native host. A few explanations may exist for this occurrence: differences in promoters could affect expression, metabolic differences between the native and the recombinant strain and the signal peptide of the protein may not be systematically recognized by the *S. cerevisiae*.

*M. pulcherrima* IWBT Y1123 was grown in media containing different nitrogen sources which included casein, BSA, grape juice proteins and ammonium sulphate. Reverse transcription-PCR (RT-PCR) confirmed that the expression of *MpAPr1* in IWBT Y1123 was induced when casein, grape juice proteins and BSA were used as sole nitrogen sources. The gene was not expressed in the presence of ammonium sulphate. This result was expected since the secretion of proteases in yeasts is induced when protein is the only available nitrogen source (Banerjee et al., 1991; Dabas and Morschhäuser, 2008). Expression of the gene was the highest when grape proteins were the sole nitrogen sources, as demonstrated by RT-PCR. The accurate profiling of the *MpAPr1* gene expression by qRT-PCR in the presence different nitrogen sources will shed some light on the effect these nitrogen sources have on the growth of the yeast. Protease activity in the presence of grape juice proteins was also confirmed by zymography, but not with casein or BSA. A possible explanation for this could be that the concentration of the protease in the gel was too low for activity to be detected, which correlates to the lower expression of the gene in the presence of casein and BSA compared to the expression induced by the grape juice proteins. Protein bands corresponding to the expected size of the putative protein was excised from SDS-PAGE gels and analysed by nano-LC-MS/MS. The bands from the casein-induced culture and the grape protein-induced culture had 21.3% and 20% identity with the putative MpAPr1 protein sequence.

The presence of *MpAPr1* was confirmed in 12 other *M. pulcherrima* strains by PCR. Plate assays revealed that the strains did not have equal activity, as indicated by the size of the halo surrounding the colonies. No correlation could be made between putative protein sequences and the level of activity displayed on plates. Investigating the sequences upstream and

downstream of the gene-encoding sequences may provide some insight into the differences in expression.

### 4.2 Conclusions and future prospects

The aim to isolate and characterize aspartic proteases encoding genes from non-*Saccharomyces* yeasts isolated from grape must, as set out in Chapter 1, has been achieved. The results obtained correspond well to existing data regarding aspartic proteases secreted by yeasts (Rao et al., 1998). Some of the grape juice proteins have been hydrolysed when cultured with *M. pulcherrima* IWBT Y1123. This illustrates the potential applicability of this yeast and /or its secreted proteases. However, further investigations are needed to test whether the protease is able to reduce haze formation and also whether the proteolytic activity will impact on the assimilable nitrogen content and aroma profile of the wine. Some wine proteins are indeed known to be insensitive to proteolytic attack due to their globular structure (Waters et al., 1992). It has been suggested that partially 'opening' up the protein structure will expose peptide bonds to be hydrolysed by proteases. One such proposal was suggested by Pocock and co-workers (2006), who demonstrated that heat treatment together with proteolysis may reduce protein haze.

This study has extended our understanding of how some yeasts may survive and even flourish in wine which is characterized by low pH, low temperatures and the presence of a number of inhibitors such as ethanol and polyphenols. Extracellular proteolytic activity that is adapted to the acidic environment may aid in nutrient supply for the yeasts.

Future prospects should include purification of the enzymes, MpAPr1 and CaAPr1, e.g. by anion-exchange chromatography and gel filtration, and biochemical characterization. This should include determination of the activity range with regard to pH and temperature. The effect of different potential inhibitors on the activity of the enzymes should also be tested, e.g. ethanol, sulphur dioxide and polyphenols. The activity of the enzymes should also be tested in wine to determine its applicability in wine biotechnology.

Mixed fermentations of *M. pulcherrima* IWBT Y1123 with *S. cerevisiae* may also be envisaged to determine its influence on protein stability, wine aroma, and the interaction between the two yeast species. By releasing proteases able to breakdown proteins present in grape must, *M. pulcherrima* may advantageously affect the fermentation kinetics by increasing the available assimilable nitrogen content for the growth of *S. cerevisiae* during alcoholic fermentation. Sequential fermentations of *M. pulcherrima* species with *S. cerevisiae* have shown positive results with regards to an increase in fruity and floral aroma in wine (Jolly et al., 2003; Rodríguez et al., 2010). The wines produced from sequential fermentations had higher concentrations of aromatic compounds such as higher alcohols, esters and terpenols compared

to fermentations where only *S. cerevisiae* was inoculated in the grape must. Commercial cultures of combined *S. cerevisiae* and non-*Saccharomyces yeasts* are already available on the market. Such an example is of *S. cerevisiae* with *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* from Viniflora® HARMONY.nsac. This combined culture is supposed to produce a wine with a richer and rounder flavour with enhanced fruity aroma notes (Jolly et al., 2006). Further investigations are required to determine the potential commercial value of *M. pulcherrima.*

### 4.3 References

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